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OF
PROGRAM ACTIVITIES
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
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Office of
the Director

NATIONAL INSTITUTES OF HEALTH
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
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The mission of the National Heart, Lung, and Blood Institute is to:

—conduct and support research on the heart, blood vessels, lungs, and on the diseases that affect them;

—develop and evaluate new or improved methods of prevention, detection, diagnostic evaluation, and treatment for these diseases;

—encourage widespread application of proven new techniques by the research and medical communities;

—provide support for the training of research workers, clinicians, and teachers in the cardiovascular, blood, and pulmonary fields; and

—provide information on research and clinical advances arising from Institute programs to health professionals and to the general public

Toward fulfillment of its mission, the Institute plans, directs, and coordinates activities that range across the whole biomedical research spectrum: basic research; applied research and development; clinical investigations; clinical trials; demonstration projects; and prevention, education, and control activities. Highlights of progress resulting from some of these activities are briefly summarized in the sections that follow.

Heart and Blood Vessel Diseases

Arteriosclerosis, a blood-vessel disease almost ubiquitous among American adults, is often the underlying cause of a variety of disabling or lethal cardiovascular conditions, including angina pectoris, acute heart attacks, sudden cardiac death, and strokes. Basic research on arteriosclerosis has included studies on the mechanisms whereby blood lipids such as cholesterol invade the innermost layers of the arterial wall and accumulate there to form the early lesions of the disease.

Animal studies suggest that a compound called dermatan sulfate may play a role in this process. A component of the ground substance that provides a matrix for the collagen and elastin fibers of arterial connective tissue, dermatan sulfate has a high affinity for lipids and preferentially binds them. NHLBI grantees noted increased concentrations of dermatan sulfate in the arteries of animals when they were fed atherogenic diets. They suspect that dermatan sulfate may act as a lipid trapping agent in the development of arteriosclerotic deposits.

Studies in non-human primates have shown that during early stages of arteriosclerosis, when the deposits were still mainly lipid, lesions could be made to regress by measures that sharply reduced blood lipid levels. But as

arteriosclerotic lesions mature, scar tissue may be laid down in and around the deposits and they may become encrusted with calcium and other substances deposited from the blood. Subsequent animal studies have indicated that lesions induced gradually over prolonged periods--as probably occurs in human arteriosclerosis--contain more scar tissue and are less responsive to lipid-lowering measures. It appears that the older and more fibrous the lesion gets, the more resistant it becomes. Less and less regression can be achieved and the rate of that regression becomes slower and slower.

Blood platelets have been implicated both in the development of arteriosclerosis and in clotting complications of the disease. Blood platelets adhering to the blood vessel wall, particularly at injury sites, may take part in the buildup of arteriosclerotic deposits. Studying pigs with von Willebrand's disease, a bleeding disorder in which platelet adhesiveness is reduced, NHLBI grantees found these animals highly resistant to the arteriosclerosis normally induced by high-cholesterol diets, developing less extensive, less severe lesions than did normal pigs. The findings raise the possibility that agents capable of reducing platelet stickiness--though not to the extent of inducing a hemorrhagic tendency--might be of value in the primary prevention of arteriosclerosis.

The aggregation or "clumping" of blood platelets appears to be an early step in the formation of blood clots. Clots forming in diseased arteries or migrating as emboli to plug vessels elsewhere in the cardiovascular system are often the immediate cause of heart attacks, strokes, and other life-threatening manifestations of arteriosclerosis.

Platelet aggregation can be inhibited by various drugs, including aspirin, and a growing body of evidence indicates that such agents can confer significant protection against thromboembolic episodes.

Aspirin inhibits platelet aggregation by inactivating the platelet enzyme cyclooxygenase, which participates in the production of thromboxane A2, a powerful aggregating agent and blood vessel constrictor. However, the blood vessel wall also contains a cyclooxygenase, and here it is involved in the production of prostacyclin. A platelet deaggregator and blood vessel dilator, prostacyclin is thought to protect arteries against arteriosclerosis and intravascular clotting. It thus appeared that whatever protection against thromboembolism aspirin conferred by inactivating platelet cyclooxygenase might well be negated by its effects on the blood-vessel cyclooxygenase.

Fortunately, subsequent research has established that platelet cyclooxygenase is much the more sensitive of the two to inhibition by aspirin. Work in progress is concerned with establishing dosages of aspirin that can halt thromboxane A2 production by platelets without hindering prostacyclin production in the blood vessel wall.

NHLBI scientists have also investigated inhibitors of platelet aggregation as a means of improving collateral bloodflow to blood-deprived areas of heart muscle. Their working hypothesis was that ischemia might trigger sludging of platelets in collateral channels and thus impede bloodflow through them.

They found that aspirin, sulfinpyrazone, or naproxin (all so-called "antiplatelet drugs") did increase collateral bloodflow by 25-50% in dogs subjected to acute coronary occlusion and also reduced the threat of arrhythmias during the post-attack period. However, the increased collateral bloodflow resulting from these agents, though beneficial, was not sufficient to salvage significant amounts of heart muscle threatened by ischemia. None of the agents had any apparent effect on the amount of heart muscle destruction resulting from the coronary occlusion.

Women enjoy relative protection against heart attacks during their reproductive years; and, though this protection diminishes after menopause, their heart-attack rate remains lower than that for men into the seventh decade. But for women who experience heart attacks, both the immediate and long-term prospects are poorer than those of male heart-attack patients, according to recent findings from the Framingham Study. Among patients aged 30-79, mortality within the first 30 days after onset of first heart attacks was 47% for women versus 38% for men. Among patients surviving their first year, subsequent mortality rates were 7.0% per year for women and 5.1% for men.

Epidemiological evidence indicates that blood levels of high-density lipoproteins (HDL) are inversely related to risk from coronary heart disease: the higher the levels of HDL, the lower the risk and vice versa. Factors associated with lower HDL levels have included obesity, cigarette smoking, and a high dietary intake of sugar, whereas moderate alcohol consumption appears to raise HDL levels.

People who exercise regularly tend to have higher HDL levels than do sedentary people. But physically active people also tend to be leaner and may differ in other respects from their sedentary counterparts, so that it has not been ascertained whether exercise per se affects plasma HDL.

According to NHLBI scientists, it does not. They examined the effects of a physical training program in young adults over a six-week period, during which the subjects consumed a constant composition diet also calculated to hold their weight steady. With diet and weight thus controlled, neither total HDL nor HDL subfractions were altered by the training program.

Significant narrowing of the left main coronary artery (the chief source of blood to the left ventricle) has been considered a major indication for coronary artery bypass surgery, since available evidence indicates that the procedure prolongs life in such patients. NHLBI studies indicate that exercise stress testing provides a highly reliable screening test for identifying these patients, even if they are asymptomatic or have only mild symptoms.

In the NHLBI series, only 7% of patients found to have left main coronary artery disease by coronary angiography had failed to show indicative ECG changes during stress testing. The scientists believe that stress testing could reduce the need for diagnostic angiograms by 50% among mildly symptomatic patients while still identifying 90% or more of patients with left main artery disease requiring angiograms as a possible prelude to coronary bypass surgery.

The Institute supports numerous studies concerned with development of techniques for precisely locating and quantifying threatened, damaged, or destroyed areas of heart muscle after acute heart attacks. Such techniques could be highly useful to clinicians, both in diagnosis and also in evaluating the effectiveness of treatment, especially therapeutic measures aimed at limitir or reducing permanent heart damage occurring during the early hours or first fe days after the attack.

In animal studies, NHLBI grantees have experimented with I-131 labeled fragments of antibodies to myosin, one of the two contractile proteins of heart muscle. The antibodies exhibited a high affinity for the myosin of necrotic heart tissue and selectively labeled infarcted areas, but their buildup was slow when the fragments were administered intravenously. However, when administered directly into the coronary arteries via catheter following induced heart attacks, the labeled fragments rapidly accumulated in infarcted areas and produced high quality scans within 30 minutes. The fragments did not promote arrhythmias or have any deleterious effects on functional heart muscle.

Although the technique is still experimental, the grantees feel that it might find clinical application in the following situations: 1) during coronary angiography in patients with unstable angina to define possible regions of infarction; 2) to define areas of permanent heart damage in conjunction with therapeutic measures to reduce infarct size; and 3) in conjunction with coronary bypass surgery to define any heart muscle injury occuring in the course of the operation.

The Institute is currently supporting several large-scale clinical trials evaluating measures for the primary or secondary prevention of arteriosclerosis and its complications. The goal of the primary prevention studies is to avert o delay the onset of crippling or potentially lethal manifestions among persons at high risk by timely interventions against modifiable factors known to increas vulnerability to premature arteriosclerosis. The secondary prevention studies seek to reduce disability and improve long-term survival among people who have already experienced serious clinical manifestations of the disease.

The Coronary Primary Prevention Trial involves 4,000 men with Type II hyperlipoproteinemia (one of the major metabolic disorders characterized by elevated blood cholesterol) but with no clinical evidence of coronary heart disease at entry into the study. It is evaluating a cholesterol-lowering diet or the same diet supplemented with cholestyramine (a cholesterol-lowering drug) in reducing expected rates of morbidity or mortality from coronary heart disease in this high-risk group.

The Multiple Risk Factor Intervention Trial involves nearly 13,000 men at heightened risk of coronary heart disease (CHD) because of various combinations of elevated blood cholesterol, elevated blood pressure, and/or cigarette smoking. Any one of these factors roughly doubles CHD risk, and the presence of all three may run it up by a factor of ten or more. We are hopeful that systematic modification of these factors over a 6-year period will produce substantial reductions in that risk and will be reflected in lowered CHD mortality rates.

The Aspirin Myocardial Infarction Study sought to determine whether daily doses of aspirin could reduce the threat of recurrent heart attacks and the numbe of heart attack deaths among 4,200 subjects who had previously experienced one or

more such attacks. A high proportion of heart attacks--whether initial episodes or recurrences--are precipitated by clotting phenomena in the coronary arteries, and it was hoped that aspirin, an inhibitor of platelet aggregration, would protect against such complications. The clinical phase of the study has been completed and the results are currently being readied for publication.

The Beta-Blocker Heart Attack Trial is evaluating another type of drug that may provide long-term protection to heart attack survivors. Propranolol, the drug being tested, has a number of potentially beneficial effects: it reduces heart rate and blood pressure, reduces the cardiac workload, is a blood vessel dilator, and protects against certain types of arrhythmias. Recruitment of participants for this trial is underway toward the goal of enrolling 4,200 patients by the middle of 1980.

The Multicenter Investigation for the Limitation of Infarct Size (MILIS) is investigating the effectiveness of propranolol or the enzyme hyaluronidase in salvaging threatened, but still viable heart muscle when infused early after the onset of acute heart attacks. It is hoped that either or both agents will reduce mortality from acute heart failure or cardiogenic shock--complications usually stemming from massive heart muscle damage sustained during or after the attack--and may also reduce residual disability after recovery. Entry of patients into the study began August 1978; 1500 will eventually participate.

The Coronary Artery Surgery Study is comparing medical management of advanced coronary heart disease with coronary bypass surgery, a procedure that has come into widespread use during recent years for the treatment of intractable or unstable angina pectoris. Among still controversial questions that this study will attempt to answer: 1) Does the procedure improve the patient's life expectancy? 2) Does coronary artery bypass reduce the heart attack risk of patients with advanced coronary obstructions or the risk of recurrent attacks in patients who have previously experienced them? 3) What is the quality of life over an extend period for patients undergoing the procedure as compare to those receiving only medical therapy?

A technique called percutaneous transluminal coronary angioplasty may provide an alternative to coronary bypass surgery in some patients with coronary heart disease. In patients with a well localized, non-calcified lesion narrowing a single coronary vessel, a balloon-tipped catheter may be passed through the site of obstruction under fluoroscopic guidance, then inflated briefly. The sausage shaped balloon, when properly positioned and inflated, compresses the malleable lesion against the blood vessel wall, thereby increasing the size of the blood channel.

Clinical experience with the technique is limited--probably about 300 procedures worldwide thus far--so that both its benefits and attendant risks are difficult to quantify. However, it has appeared sufficiently promising for NHLBI to establish a registry to facilitate further evaluation of its effectiveness and to define its limitations. Additional laboratory and clinical research with the technique is also being encouraged.

In coronary artery bypass surgery, obstructions in coronary vessels are usually bypassed by splicing segments of saphenous vein (obtained from the patient's legs at time of operation) into the coronary artery beyond the major site of obstuction, then splicing the other end of the graft into the aorta. Alternatively, the left and right internal mammary arteries (which normally supply blood to the chest wall) may be mobilized as bypass vessels. Artificial blood vessel grafts had not been used because even slight blood incompatibilities of most graft materials, together with the small sizes required in coronary bypass surgery (usually less than 5 mm. in diameter), made graft closure by clots almost inevitable.

However, NHLBI grantees report using artifical grafts of polytetrafluoethylene to bypass coronary artery obstructions in five patients for whom no other alternatives were feasible because of earlier vein stripping operations and/or obstuctions unreachable with internal mammary bypasses. To help insure continued patency, the ends of each graft were expanded into a "cobra head" shape before splicing, and meticulous technique was employed in suturing them into place. Followup studies 9 to 14 months after surgery showed 4 of the 5 grafts still functional. These preliminary results are promising, though followup for much longer periods will be necessary before polytetrafluoethylene grafts can be recommended as an alternative to saphenous vein or mammary artery grafts in coronary bypass procedures.

Hypertension is another priority target of NHLBI programs because of its high prevalence in the U.S. population (an estimated 35 million Americans have it) and because of its importance as a risk factor for heart attacks, strokes, congestive heart failure, and kidney failure.

Essential hypertension is usually considered a disease of adult life because it most often becomes manifest during the third decade or later. However, growing evidence suggests that forerunners of the disease may be identifiable much earlier.

Studies among children and adolescents indicate that blood pressures higher than the norm for children of the same age group tend to persist at these higher levels as the child grows older, possibly moving into the hypertensive range during adult life.

Urinary kallekrein levels may also be an early indicator of hypertension risk. Kallekreins are enzymes that participate in the production of kinins, many of which are potent blood vessel dilators. The kallekrein-kinin system is suspected of playing an important, though still unspecified role in blood pressure regulation. In adults, urinary levels of kallekreins appear to be inversely related to blood pressure levels: the lower the urinary kallekrein levels, the higher the mean blood pressure and vice versa. The same inverse relationship appears to hold among children and adolescents. And, as with blood pressure levels, low urinary kallekrein levels during childhood tend to persist as the child grows older, possibly indicating a heightened risk of developing hypertension during adult life.

Also assigned an important role in blood pressure regulation and in some forms of hypertension is the renin-angiotensin system. Renin, an enzyme produced by the kidney, acts on a plasma protein to generate angiotensin I. Angiotensin I is relatively inactive, but as it traverses the lungs (and possibly other tissues) it is exposed to a converting enzyme in cells lining the pulmonary blood vessels, generating angiotensin II. A powerful blood vessel constrictor, angiotensin II is the chief culprit in renal hypertension, a major factor in the malignant phase of essential hypertension, and is suspected of involvement in earlier stages of the disease as well as later complications, such as congestive heart failure.

Recently, drugs have been developed that impede angiotensin II production by inhibiting the converting enzyme that generates it from angiotensin I. One of the most promising of these drugs, Captopril, had performed well in the treatment of both renal and essential hypertension in limited clinical trials reported earlier by NHLBI grantees, Now other grantees report that Captopril may also prove valuable in the treatment of chronic congestive heart failure that responds poorly to other therapeutic measures.

With the development of congestive heart failure, diminished heart output elicits widespread blood-vessel constriction, which forces the heart to pump against a higher resistance. Another factor in this higher resistance is thought to be increased stiffness of the blood-vessel walls, possibly due to their increased sodium content. The renin-angiotensin system may well be involved in both phenomena, since angiotensin II is not only a blood-vessel constrictor but also a potent stimulus for the production and release of aldosterone, a salt-retaining hormone from the adrenal cortex.

In any case, impedence of angiotensin II production by Captopril brought about substantial reductions in systemic and pulmonary vascular resistance, decreases in arterial pressure, improved heart performance, and clinical improvement in the patients receiving the drug.

The renin-angiotensin-aldosterone system affects and is affected by dietary salt. A low sodium intake results in increased plasma renin activity, generating more angiotensin II and thereby stimulating increased production and release of aldosterone. Aldosterone, in turn, promotes sodium conservation by the kidneys. Recent research suggests that these effects of dietary salt may be mediated through larger, biologically inert molecules (sometimes called "big renins") that serve as precursors for the smaller, biologically active renins secreted by the kidneys. With sodium depletion, the concentrations of the larger molecules decrease as concentrations of the smaller renins and plasma renin activity increase.

A high salt intake is thought to contribute to the development of essential hypertension in some people, and here again the renin-angiotensin-aldosterone system appears to be involved. High salt intakes normally reduce plasma renin activity and thereby curb angiotensin and aldosterone production. Without the aldosterone stimulus, the kidneys excrete sodium

in excess of bodily needs. But the response of this system is apparently blunted in some people, said to be "salt sensitive." In such people, persistently high salt intakes are thought to increase their vulnerability to essential hypertension.

Race may be a factor in salt sensitivity. Studies comparing the responses of normotensive white or black subjects to an administered salt load indicated that, as a group, the whites excreted the excess salt more readily. Plasma renin activity was less effectively suppressed by salt loading in the black subjects and excess sodium was retained longer. The findings raise the possibility that the greater salt sensitivity of blacks could well be a factor in their higher prevalence of hypertension, nearly twice that of whites in the U.S.

Thiazide diuretics promote salt excretion by the kidneys and also exert a direct blood-pressure-lowering effect. Thiazides alone can often control mild hypertension, and these agents also potentiate the effects of other blood pressure drugs. As a result, they are a component of most antihypertensive regimens.

But thiazides also tend to raise serum uric acid levels, and this is a potential source of problems for the 25% of hypertensive patients who also have hyperuricemia. Approximately 12% of such patients may develop gout on regimens that include thiazides. Adding a uricosuric agent, such as probenecoid or allopurinol, to the regimen may eliminate this risk, but also makes the regimen a bit more complicated to follow.

Currently undergoing clinical trials is a new diuretic agent, ticrynafen, which reduces serum uric acid. In trials comparing it with a thiazide, NHLBI grantees found that both agents produced very similar effects on the patients' blood pressure; but whereas their uric acid levels rose with thiazide therapy, they fell with ticrynafen therapy. The results suggest that ticrynafen may find a most useful niche in the management of hypertension accompanied by hyperuricemia.

The Hypertension Detection and Followup Program involved 11,000 patients followed for five years to assess the impact of controlling moderately elevated blood pressure in reducing morbidity and mortality from hypertension-associated cardiovascular disorders. The clinical phase of the program was completed this year and the results are being analyzed and prepared for publication. They will compare the morbidity and mortality experience in an intensively treated stepped-care group with that of patients referred to their usual sources of medical care.

Coordinated by NHLBI, the National High Blood Pressure Education Program, since its inception in 1972, has carried out a wide range of activities concerned with alerting health professionals and the general public to the wide prevalence of hypertension, the dangers that uncontrolled hypertension poses to the victim's health and life, and the benefits of early detection and adequate treatment. The program enjoys the support and cooperation of numerous federal agencies, 150 or more national organizations, and virtually all state health departments. Its success

has been reflected in sharp decreases in the numbers of hypertensives unaware of their disease, by dramatic increases in patient visits for hypertension and in prescriptions written for blood-pressure drugs, and by substantial increases in the numbers of hypertensive patients whose blood pressure is under adequate control.

In 1977 the Institute provided funds to initiate large-scale demonstration projects in hypertension education, screening, and control throughout the states of California, Connecticut, Maryland, and South Carolina. Similar projects have since been funded in Georgia, Maine, and Michigan. The goals of these projects are to provide expansion and effective coordination of ongoing education, screening, and treatment activities so as to avoid needless duplication or fragmentation of effort while insuring the availability of effective methods of hypertension detection and control, not only to communities throughout the state but to rural areas as well. It is also hoped that the experience of these states may provide encouragement and possible models for other states desiring to undertake similar programs.

The Institute is also funding demonstration projects concerned with hypertension detection and control in the work setting at four Ford plants in Southeastern Michigan, five Westinghouse plants around the U.S., and among Maryland state employees in Baltimore. Pilot evaluation studies of hypertension control in communities with a high prevalence of the disease have been initiated in Detroit; Berkeley, California; and also in rural areas of North Carolina, Georgia, and Kentucky.

NHLBI scientists have found that a frequent cause of sudden death among athletes or other young, vigorous, seemingly healthy people is hypertrophic cardiomyopathy (HCM). Evidence of HCM was found at autopsy in nearly half of 29 athletes aged 14-30 years who died suddenly and unexpectedly and also in the majority of 25 other patients in whom sudden death was the first overt manifestation of disease. The usual features of HCM are marked thickening of the ventricular septum and the presence of numerous disorganized cardiac muscle cells, not only in the septum but also in the free wall of the left ventricle. This diffuse cellular disorganization is thought to predispose to the arrhythmias that are usually the cause of sudden death in HCM patients.

The electrocardiogram is usually abnormal in HCM and the thickening of the ventricular septum is readily detectable by echocardiography. But neither test is likely to be used routinely in examining would-be athletes or team members, nor would the need for such tests be obvious in a young, seemingly robust athlete who just happens to have HCM.

The drug of choice for treating HCM is propranolol. In the obstructive form of the disease, surgery may provide relief of symptoms if propranolol is ineffective. But until recently there had been no satisfactory therapeutic alternative for the patient with non-obstructive HCM who did not respond well to propranolol.

NHLBI scientists report that the calcium antagonist verapamil may provide that much-needed alternative, both in obstuctive and nonobstructive

HCM. Comparing verapamil and propranolol in subjects with obstructive HCM, the scientists found both about equally effective in relieving outflow obstruction and improving exercise tolerance. Against the other major threat of HCM--cardiac arrhythmias--verapamil was highly effective against atrial arrhythmias but less consistently so against ventricular arrhythmias.

The scientists also noted that about one-third of HCM patients either could not tolerate verapmil or else did not respond to it, so that further clinical studies are needed to determine the place of this drug in the treatment of HCM.

For replacement of damaged heart valves, the Hancock porcine bioprosthesis -- a specially treated pig heart valve mounted on a prosthetic frame for ease of insertion---combines good performance characteristics with exceptionally low risk of engendering clots or emboli, thus obviating the need for anticoagulants postoperatively. The unanswered questions about this valve, as with bioprosthetic valves in general, had concerned its durability.

NHLBI studies among 69 recipients of Hancock valves--all are followed for more than 4.5 years--have disclosed late valve failures in 6 patients. The failures occurred 56-100 months after insertion and usually resulted from calcificiaton or disruption of the valve leaflets. Microscopic examination of the leaflets revealed degeneration of their collagen fibrils, calcification, and infiltration by lipid and fibrinoid substances.

NHLBI experience thus far suggests that the probable useful life of Hancock valves is 5-8 years, after which a high failure rate is likely. Henceforth, these scientists will continue to use the Hancock valve in patients sixty or older, but will select some other suitable artificial valve for use in younger patients.

Lung Diseases

Oxygen therapy is often essential in the clinical management of respiratory distress syndromes, advanced emphysema, and other lung disorders. But prolonged exposure to high concentrations of oxygen is toxic to the lung and can lead to fibrosis or other degenerative changes. The culprit in oxygen toxicity appears to be free radicals of oxygen. Such free radicals are highly reactive and may combine avidly with intracellular substances, including enzymes and other macromolecules, sometimes with disruptive effects on their functions.

Basic research in animals has disclosed that antioxidant enzymes including superoxide dismutase helps protect the lung against oxygen toxicity by converting free radicals into non-toxic products. The protection afforded by this antioxidant system is greatest in the lungs of immature animals, in whom exposure to toxic levels of oxygen elicits an increase in superoxide dismutase activity. As the animal matures, however, the responsiveness of the system diminishes, as does its

protection against oxygen toxicity.

But it appears that the antioxidant system can be activated in mature animals by endotoxin. A single dose of endotoxin protected animals against lung damage following exposure to oxygen at levels that produced severe pulmonary fibrosis or even death in untreated animals. These findings raise the possibility that other activators of this antioxidant system that are less toxic than endotoxin may be found, with possible application to the prevention of toxicity that might otherwise result from prolonged or intensive oxygen therapy.

A sonar-like technique that shows promise in pulmonary-disease screening--especially in young children--employs a transducer that generates oscillating soundwaves at the subject's mouth. The sound waves are beamed down the trachea and, as they traverse the lung airways, their reflections are picked up and analyzed. The technique can provide valuable information on airway geometry and reliably detects airway obstruction, a cardinal feature of a number of pulmonary disorders. The technique causes no discomfort to the subject, so that frequent or repeated measurements are possible.

Research evidence continues to accumulate in support of the protease-antiprotease theory of emphysema. The theory holds that the lung damage of emphsema may stem, at least in part, from local imbalances in lung tissues between enzymes capable of degrading the collagen or elastin of lung connective tissue and the antiproteases that normally neutralize them The sources of these proteases are white blood cells, especially neutrophils and alveolar macrophages, which release them in the course of their battles with bacteria and other foreign invaders of the lung. Ironically, these byproducts of lung defense mechanisms can themselves attack lung connective tissue unless inactivated.

A small segment of the population (about 0.02%) is rendered particularly vulnerable to emphysema by a marked hereditary deficiency of alpha-1-antitrypsin, one of the most important of the antiproteases. These people, homozygous for the enzyme deficiency, have alpha-1-antitrypsin levels only about one-ninth normal. (Although heterozygotes also have lower than normal enzyme levels, they are apparently still adequate to prevent any significant increase in emphysema risk.)

In homozygotes, alpha-1-antitrypsin synthesis by the liver continues, though possibly at a reduced rate. But, possibly because of structural alterations in the protein molecule, it cannot be secreted normally by the cells in which it is made. Recent NHLBI studies indicate that an androgen-like drug, danazol, somehow helps alleviate this block on alpha-1-antitrypsin release. In homozygous patients, it raised blood levels of the enzyme by 40%. However, further detailed study will be needed to ascertain whether such drug-induced increases in serum enzyme levels will appreciably decrease the emphysema risk in homozygotes or mitigate the course of the disease in those already afflicted.

The scientists have also drawn up a protocol for a clinical study to determine whether alpha-1-antitrypsin replacement is feasible in homozygotes. It calls for intravenous infusions, once a week, of partially purified alpha-1-antitrypsin in amounts equivalent to that present in two liters of normal plasma. The goal is to find out whether protective levels of the enzyme can be maintained in this fashion.

Other NHLBI studies suggest some possible reasons why cigarette smokers are at increased risk from emphysema. They showed that alveolar macrophages obtained by lavage from the lungs of smokers produced a chemotactic substance that attracts neutrophils. Macrophages from the lungs of nonsmokers could also be induced to release this substance when these cells were exposed in culture to cigarette smoke.

The findings may help explain why, in smokers, neutrophils (a prime source of proteases) constitute a higher proportion of the leukocyte population in their lower respiratory tract (2-3% versus less than 1% in nonsmokers). Moreover, the same chemotactic factor that attracts increased numbers of neutrophils to the lung also "activates" these cells for subsequent release of various substances, including proteases against both collagen and elastin of lung connective tissue.

The studies further disclosed that the lungs of smokers may have less protection against these proteases: their antiproteases against elastin-digesting enzymes were only about half as effective as those from nonsmokers.

Other NHLBI research has centered on the synthesis and metabolism of collagen in lung tissues, on the mechanisms that regulate and modulate these processes, and on disturbances resulting from various disease states that may lead to the destruction of functional lung components or else to widespread fibrosis throughout the lung.

A major site of collagen synthesis in lung is the fibroblast. NHLBI studies indicate that cyclic-AMP--a "second messenger" substance that often mediates the effects of neural, humoral, or other stimuli in susceptible tissues--is also critically involved in collagen production by these cells. In general, agents that increased cyclic-AMP levels in fibroblasts resulted in decreased collagen production. All such agents that inhibited collagen synthesis were stimulants of beta adrenergic receptors. Conversely, agents that blocked beta adrenergic receptors increased collagen production.

The scientists suspect that intracellular substances acting on beta-adrenergic receptors in the fibroblast, possibly through the intermediary substance cyclic-AMP, may regulate its collagen production. Under normal circumstances, they reason, collagen production is kept partially suppressed by endogenous stimulation of these receptors. When increased collagen production is needed (as, for example, in tissue repair) endogenous stimulation of these receptors is diminished or withdrawn, the brakes on collagen synthesis are released, and more collagen is produced.

This hypothesis suggests a possible mechanism for the fibrosis that has sometimes occurred in various tissues of patients under treatment with propranolol. This drug is a potent beta adrenergic blocker and hence a potential stimulant of collagen production. The hypothesis could also lead to fresh insights or new approaches to the prevention or treatment of fibrotic lung disorders. Collectively, these comprise 15-30% of all noninfectious diseases of the lung, excluding tumors.

Each year in the U.S., some 50,000 newborn infants develop neonatal respiratory distress syndrome (RDS). Premature infants are especially susceptible because their lungs may not have matured sufficiently to produce adequate quantities of pulmonary surfactant.

The results of animal and clinical studies have indicated that certain steroids accelerate maturation of fetal lungs. Currently, NHLBI is supporting a clinical trial to assess the effectiveness of steroids, administered antenatally to mothers, in preventing RDS among their infants at risk. The infants will subsequently be followed for 18 months to ascertain whether the steroids result in any untoward short term or long term effects.

Blood Diseases and Resources

It is now possible to identify female carriers of hemophilia with accuracies up to 90% by combining the results of coagulation tests for normal factor VIII with immunological tests which also react with the abnormal variant that does not participate in clotting. If the known carrier becomes pregnant, it is also possible to identify the sex of the unborn fetus by amniocentesis. And now, NHLBI grantees report, if the fetus is male (and thus has a 50-50 chance of being hemophiliac) it can be determined during the second trimester whether the fetus is normal or has inherited the disease.

The test entails using a fetoscope to obtain a fetal blood sample from the placenta, then assaying its content of normal factor VIII. The procedure requires skilled fetoscopy and meticulous laboratory technique, since the blood sample is usually considerably diluted with amniotic fluid. However, using their technique in 14 carriers, the grantees correctly identified 7 of 7 hemophiliac fetuses (confirmed at autopsy after induced abortions); and of 7 other fetuses identified as normal, all five that had been born at the time of the grantees' report had proved to be so.

The same technique can be employed to identify fetuses who inherit von Willebrand's disease. This disorder, like hemophilia, is characterized by a factor VIII deficiency, but it also involves a platelet defect that may contribute to the associated bleeding tendency. Von Willebrand's disease also differs from hemophilia in that the flawed gene is a dominant one and may be transmitted by either parent to offspring of either sex.

Von Willebrand's disease appears to be the most common of the hereditary clotting factor deficiencies. Fortunately, in most affected persons factor VIII levels are sufficiently high to prevent spontaneous hemorrhage, though excessive bleeding may follow trauma, surgery, childbirth, and the like. But because the expression of the genetic flaw is highly variable, some von Willebrand's patients exhibit very low factor VIII levels and pronounced hemorrhagic tendencies.

The new technique can thus be immensely helpful to prospective parents who are carriers of these diseases and who may find themselves faced with some wrenching decisions.

At the opposite pole from the hemorrhagic disorders are clotting complications that are often directly responsible for the disabling or lethal manifestations of heart and blood vessel disorders. Recent NHLBI-supported research has been seeking rapid, reliable techniques for the detection and evaluation of intravascular clotting by measuring blood levels of various substances participating in coagulation or generated as byproducts of the process.

One such byproduct is fibrinopeptide A, which is liberated during the formation of fibrin clots. The appearance in blood of significant quantities of this peptide is strongly indicative of intravascular clotting, even in the absence of overt clinical signs or symptoms. It is hoped that continued development and refinement of such techniques will permit recognition in high-risk patients of "clinically silent" thrombo-embolic episodes or potentially serious clotting problems in the making so that appropriate therapeutic measures can be initiated promptly to head them off or else to halt their progress.

NHLBI research on sickle cell disease is pursuing a number of promising lines of inquiry toward the goal of developing improved means of prevention or treatment.

One of these is concerned with the switchover in hemoglobin synthesis that occurs late in fetal life. The hemoglobin molecule 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. Unfortunately, in sickle cell disease, the beta chains are flawed; and the resulting molecules of sickle hemoglobin tend to aggregate into a rigid gel under various conditions, distorting the red cell into the characteristic sickled shape.

If fetal hemoglobin synthesis could be stimulated and maintained in sickle cell patients, many of the problems of the disease would disappear along with the troublesome beta chains. Even achieving a mix of fetal and sickle hemoglobins by a partial replacement of the latter could help a lot.

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 (though not, so far, a switch back from adult to fetal hemoglobin synthesis).

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, scientists hope to learn how the gamma globin gene is "turned off" and the beta globin gene "turned on" —and vice versa.

Another line of inquiry is concerned with developing means of increasing the affinity of sickle hemoglobin for the oxygen that it carries. Under normal circumstances, hemoglobin readily takes on oxygen in transit through the lungs and subsequently surrenders it just as readily to the tissues that need it. However, deoxygenated sickle hemoglobin is particularly prone to aggregation. So, the scientists reason, if a small proportion of the hemoglobin could be induced to hold on to its oxygen a trifle more tenaciously sickle cell crises might be averted or their severity substantially reduced.

Measures to increase the oxygen affinity of sickle hemoglobin might reduce somewhat the efficiency of the red cell in delivering its oxygen to tissues; but the tradeoff would be that unsickled cells could reach the tissues easily, whereas sickled cells often have trouble negotiating

the smallest blood vessels and frequently create "log jams" that impede blood flow or may cut it off completely.

Other investigations are employing x-ray diffraction and other techniques of physical chemistry to examine the molecular structure of sickle hemoglobin and to locate the molecular sites participating in the aggregation of these molecules into a rigid gel, as occurs in sickling.

By learning more about these "active" sites and the manner of their bonding to other sickle hemoglobin molecules, it may become possible to prevent sickling by introducing other small molecules that selectively tie up these sites.

In Cooley's anemia (beta thalassemia), as with sickle cell anemia, the problem is in the adult hemoglobin beta chain, which is not made in sufficient quantities. An excess of alpha chains builds up and precipitates in the red cell, blighting it and so reducing its lifespan. Hence the results are anemia that, at present, can be corrected only by repeated blood transfusions.

As fetal hemoglobin contains no beta chain, the research cited earlier seeking means of reinstating and maintaining fetal hemoglobin synthesis in sickle cell disease, if successful, could eliminate or mitigate many of the clinical problems of Cooley's anemia as well.

Iron overloading remains a serious problem of Cooley's and other transfusion-dependent anemias. The iron-chelating agent desferrioxamine, continuously infused under the skin by a small clockwork pump for 6-12 hours a day, has been shown capable of preventing excessive iron buildup in younger patients and of halting or even reversing iron deposition in older ones. Some patients, who had been sliding steadily into congestive heart failure because of iron deposition in heart muscle, improved dramatically with desferrioxamine therapy. Meanwhile, other iron chelating agents are under study, including one called rhodotorulic acid that may prove to be even more effective than desferrioxamine.

NHLBI through its Division of Blood Diseases and Resources carries the responsibility for research into biomedical as well as logistical and managerial aspects of the national blood resource. In both areas considerable progress has been made during the past year.

DBDR has sponsored the development of artificial blood substitutes, an important research area that promises to bear fruit in the near future. It was shown some time ago that synthetic chemicals known as fluorocarbons, when injected into the blood stream, can carry oxygen to the tissues in a manner somewhat anologous to the hemoglobin contained in the red corpuscles. NHLBI-supported investigators showed that completely exsanguinated animals, when perfused with a fluorocarbon preparation known as fluorodecaline, could survive for an indefinite period of time. The application of this achievement to human medicine requires the development of stable emulsions of fluorocarbons that are non-toxic and non-carcinogenic and will leave the body within a short time after they have served their prupose of sustaining life. Previously some eighty compounds were synthesized under NHLBI sponsorship. Four of these were found to have highly desirable properties and are currently being tested. The use of synthetic blood substitutes would be a major advance in transfusion therapy. It would obviate many of the hazards of blood transfusion, notably transfusion-transmitted hepatitis and transfusion reactions due to blood group incompatibility. It should be especially helpful in tiding patients with massive blood loss over the period required for the procurement and cross-matching of compatible blood and possibly make many transfusions altogether unnecessary. It can be expected to reduce the strain on the national blood resource. In addition, evidence is beginning to accumulate to the effect that fluorocarbons are highly suitable for the perfusion and extended perservation of organs for transplantation.

The Divsion has a major role in the implementation of the National Blood Policy, and in this connection has supported a number of Task Forces of the American Blood Commission. One result has been the creation, with support of DBDR/NHLBI, of a National Blood Data Center, currently entering its second year, designed to serve as an instrument of the blood services complex for the ascertainment of the capabilities and needs of blood banks in the United States. This goal was formulated in 1973 and is now being met. Progress was also made by the Task Force on Regionalization of the ABC, which has instituted a program of recognition for Regions which meet criteria for complete blood services previously formulated.

Fourteen Regions have now fully satisfied these criteria, 12 have been provisionally recognized, 11 are under consideration, and 20 have indicated their intent to participate. The goal is to assure optimal blood services for every person in the country.

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Division of Heart
& Vascular Dis.

DIVISION OF HEART AND VASCULAR DISEASES
ANNUAL REPORT

General Mission

A. The Division of Heart and Vascular Diseases has continued the broad mandated responsibilities established in 1948 by the National Heart Act which created the National Heart Institute, the predecessor of the National Heart, Lung, and Blood Institute, as the primary federal agency responsible for the conduct and support of research relating to the cause, prevention, methods of diagnosis and treatment of diseases of the heart and blood vessels.

B. Additionally, under Public Law 95-622 the extension of authorization for research training through National Research Service Awards, the Division has authority to make grants for pre- and post doctoral training of individuals to undertake research in the causes, diagnosis, prevention and treatment of cardiovascular diseases.

Magnitude of the Problem

C. Three of the ten leading causes of death in the United States are heart and blood vessel diseases: heart disease, cerebrovascular disease, and arteriosclerosis (Table 1). While these numbers indicate that cardiovascular disease is a large chronic disease problem, trends in death rates show that substantial improvements have taken place: 1. The age-adjusted death rate for these diseases combined has been downward for more than 30 years; 2. Recent decreases have been especially steep; 3. The rate for coronary heart disease, the largest component, turned in the 1960's from an upward to a downward trend; 4. Rheumatic, cerebrovascular, and hypertensive diseases continue very large declines; and 5. Most decreases for cardiovascular diseases exceed percent declines for other causes of death. In fact, the average annual decline in the cardiovascular death rate since 1970 has been 3%, more than twice the decline for all other causes of death combined (Figure 1).

Progress Toward Achieving Objectives

The Division has maintained its strong base of research support for investigations into the causes of heart and vascular diseases including research in prevention and in improved therapies. High priority also has been given to provide training for promising young investigators including minority professionals to continue manpower development for research in cardiovascular diseases. Because of the ongoing commitments to fund existing research programs only three modest new initiatives could be implemented. These were:

1. Preventive Cardiology Academic Awards
2. RFA in the Effect of Diet on the Metabolism and Blood Levels of High Density and Other Lipoproteins
3. Procurement of Standard Reference Materials for Evaluating Blood Material Interactions for Circulatory Assist Devices.

TABLE 1

MORTALITY FORM THE TEN LEADING CAUSES OF DEATH;

U.S., 1977

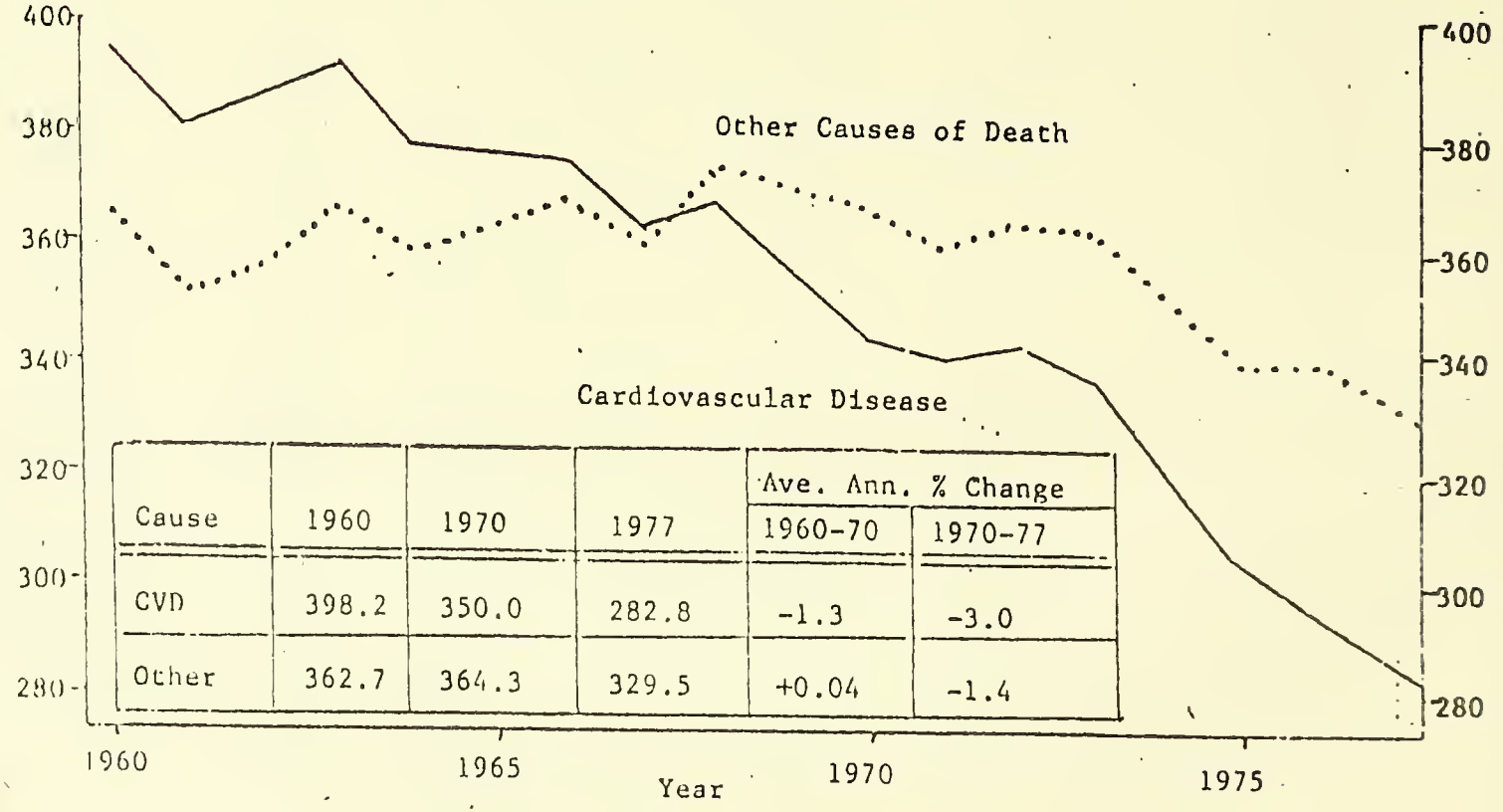
Cause of Death	Number	Rate per 100,000 Population
Total	1,899,597	878.1
1. Diseases of the heart	718,850	332.3
2. Malignant neoplasma	386,686	178.7
3. Cerebrovascular diseases	181,934	84.1
4. Accidents	103,202	47.7
5. Influenza and pneumonia	51,193	23.7
6. COPD [a]	44,651	20.6
7. Diabetes	32,989	15.2
8. Cirrhosis of the liver	30,848	14.3
9. Arteriosclerosis	28,754	13.3
10. Suicide	28,681	13.3
All other causes	291,809	134.9

a. This includes deaths from emphysema, chronic bronchitis, and chronic obstructive pulmonary disease without mention of emphysema or chronic bronchitis. The official NCHS ten leading causes of death does not include COPD.

SOURCE: Prepared by NHLBI; data from NCHS.

Figure 1

Death Rates for Cardiovascular Diseases
and Other Causes of Death*
U.S., 1960-1977



Cause	1960	1970	1977	Ave. Ann. % Change 1960-70	Ave. Ann. % Change 1970-77
CVD	398.2	350.0	282.8	-1.3	-3.0
Other	362.7	364.3	329.5	+0.04	-1.4

* Age-adjusted to U.S. population, 1940.
SOURCE: Prepared by NHLBI. Data from the National Center for Health Statistics.

Highlights of Accomplishments

1. Arteriosclerosis

Research in arteriosclerosis comprises the largest segment of scientific activities supported by the DHVD. The scope of research includes fundamental and metabolic investigations into the etiology, pathogenesis, induction and regression of the disease. Epidemiological, pathological and biochemical studies are also being conducted to identify factors that influence the development and severity of this prevalent disease. The role of diabetes in atherogenesis is also receiving additional emphasis.

Examples of investigations and highlights of accomplishments in arteriosclerosis research supported by branches of the DHVD are:

a. Most studies of atherogenesis have revolved around the details of systemic lipid metabolism with a growing appreciation of the complexities of lipoprotein structure and function. The past year has seen the recognition that endogenous sterol elevation can be atherogenic and that chylomicron remnants may also be atherogenic.

b. The question of lesion regression in adult humans remains to be demonstrated unequivocally although suggestive evidence has been obtained. On the other hand, it is now clear that the longer the period over which experimental lesions are formed in non-human primates, and the more fibrous they are, the slower do they regress and the more limited their regression is.

c. Both clinical experience and animal models continue to provide examples of the observation that regional vascular beds differ in their susceptibility to atherogenesis. There is a genetic component to this regional susceptibility. Particular local injuries are under study as elements in the localization of plaques, but the experimental phenomena are highly artificial, involving such injuries as endothelial denudation, regional hypertension or sympathetic denervation.

d. The mechanism by which cholesterol esters become associated with LDL is unknown. The isolation from human plasma of a transfer protein, possibly identical to apo D, which catalyzes the net transfer of cholesterol esters from HDL to LDL may have resolved this issue.

e. Increases in the concentration of dermatan sulfate in the arteries of monkeys and pigeons during respective peanut oil-induced or cholesterol-aggravated arteriosclerosis have been reported. This glycosaminoglycan has been shown by others to preferentially bind lipid and it may represent a key trapping agent for lipids during atherogenesis.

f. A study of the incidence of diabetes mellitus in the Framingham cohort showed: (1) latent diabetic men and women were extremely

obese, had greater VLDL and higher casual glucoses before the diagnosis of diabetes; (2) glucose was the best individual predictor of future diabetes in men; and (3) degree of obesity was the best predictor of diabetes in women.

2. Hypertension

Highlights of progress in hypertension are:

a. Through an interagency agreement with the National Bureau of Standards pure synthetic Angiotensin I peptide has been produced as a reference standard for research use. A high performance liquid chromatography method has been developed for Angiotensin I and is also useful for purification of other small peptides.

b. A thromboxane A2 antagonist has been synthesized and its activity independently confirmed. Unlike enzyme inhibitors, this antagonist does not affect the prostacyclin systems. Named pinane-thromboxane A2 (PTA2), this antagonist should provide a valuable research tool for studying the role of prostaglandin compounds and their relation to hypertension.

c. Purification of human renin has been accomplished by employing several affinity chromatographic systems developed specifically for that purpose which yielded an electrophoretically homogeneous enzyme representing a more than 400,000 fold purification.

d. The Brookhaven National Laboratories have reported an irreversible kallikrein inhibitor that inactivates both human and rat urinary kallikrein. This laboratory is presently exploring the activity of this compound in vivo.

e. The regional distribution of angiotensinogen in rat brain has been quantified. Angiotensiongen, the substrate for renin and precursor of Angiotensin II, was widely distributed throughout 31 brain regions. It is suspected that the angiotensinogen distribution exhibits a brain pattern similar to that observed for norepinephrine.

f. Angiotensins II and III have also been localized in rat brain. These vasopressor materials are widely distributed and evidence indicates that they may reach receptors within the central nervous system by both vascular and neuronal channels.

g. Studies of the effect of dietary sodium manipulation suggest that the inactive, large molecular weight renins are precursors of the smaller molecular weight, more active renins secreted by the kidneys. Sodium depletion decreases the quantity of the larger forms as the concentration of the active, smaller renin increases. The rate of formation of active renin from larger inactive precursors may be the biochemical mechanism by which sodium intake controls active renin levels in plasma.

h. Results of a series of studies strongly suggest that a large fraction of individuals with normal-renin essential hypertension have an alteration in the adrenal receptor for Angiotensin II. This finding may prove to be significant help in understanding volume abnormalities in patients with essential hypertension.

i. The Hypertension Detection and Follow-up Program, a clinical trial to determine the extent to which mortality from hypertension in the general population might be reduced by sustained antihypertension drug therapy has completed its planned five year follow-up of a study group of 10,940 hypertensive men and women between the ages of 30-69. At the five year anniversary of enrollment, 75.2% of the living stepped care participants were maintained under active treatment and of this group 73.3% were at or below their individual goals of reduced blood pressure. The total mortality experience of the intensively treated group as compared to those receiving regular medical care will be analyzed and prepared for scientific publication in December 1979.

j. Two urban and three rural pilot evaluation studies of community hypertension control in communities with high population prevalence of hypertension have been implemented. The urban studies in Detroit and in Berkeley, California have completed baseline surveys in their population areas and are beginning a planned program of education, referral and antihypertensive therapy. The rural studies in North Carolina, Georgia and Kentucky have just been started.

k. Three additional Statewide Demonstrations of the Impace of Statewide Coordination on High Blood Pressure Control have been funded. This expands this program to seven states: California, Connecticut, Maryland, South Carolina, Georgia, Maine and Michigan in response to Congressional interest in the problems of high blood pressure control.

3. Coronary Heart Disease

Division funding for research in coronary heart disease ranks third following arteriosclerosis and hypertension. Significant gains in knowledge relating to natural history, diagnosis and treatment, including advances in medical and surgical techniques. Epidemiological studies have continued to expand the identification of risk factors and possible protective factors as determined in prospective population studies. Primary prevention clinical trials and clinical trials to reduce mortality following myocardial infarction have made good progress. Highlights of research in coronary heart disease are:

a. A 10 year follow-up of men and women who sustained a recognized first myocardial infarction in the Framingham Study found a less favorable prognosis for women than for men. Women had higher early mortality than men in the first year. Mortality within the first 30 days, including sudden deaths was 38% for men aged 30-79 and 47% for women. After one year the mortality was 5.1% per year for men and 7.0% per year for women.

b. The Aspirin Myocardial Infarction Clinical Trial has completed its planned period of clinical study. The results are being prepared for publication. The Beta-blocker Heart Attack Trial, also concerned with the evaluation of therapy to reduce coronary heart disease deaths among persons who have had acute myocardial infarction is continuing in its recruitment phase. A goal of 4200 patients is expected to be enrolled into this clinical trial by the middle of 1980.

c. A scientific monograph on high density lipoprotein (HDL) methodology findings from 14 epidemiologically defined populations in the Lipid Research Clinics Population Studies is being prepared by the investigator group. Relationships between HDL and a wide number of factors such as physical fitness, ponderosity, socioeconomic status, ethnicity, race, diet, alcohol consumption and cigarette smoking are being analyzed, among the findings are that HDL of smokers is significantly lower than among nonsmokers, a direct relationship of alcohol consumption and HDL was confirmed and an inverse association between percent of calories as dietary sucrose was found.

d. Baseline lipid data characterizing 60,000 individuals from defined populations participating in the LRC prevalence studies have been prepared in a monograph to be published by NHLBI. A baseline paper presenting data from the participating American clinics is being published in the August issue of Circulation. Many other individual publications are being generated by the LRC groups.

4. Cerebrovascular Disease

A comparatively small level of scientific investigation is being supported directly relating to cerebrovascular disease in contrast to the areas of arteriosclerosis research and hypertension research. Some conjoint studies of the epidemiology of atherothrombotic stroke and hemorrhagic stroke are also components of most prospective epidemiological studies conducted or supported by the Division. The Hypertension Detection and Follow-up Program will also be providing the results of antihypertensive drug therapy in relation to mortality from stroke based on five years of therapeutic management. Some progress is continuing to cerebrovascular research despite limited research emphasis in this area. Highlights in this area are:

a. Aspirin and indomethacin, both of which block prostaglandin or thromboxane synthesis inhibit aggregation in pial arterioles, as indicated by a prolongation of the time required for noxious stimuli to initiate aggregation. It is thought that only the anti-aggregating properties (and not the antiinflammatory properties) of these drugs are effective in preventing the consequences of vessel injury. Thus, aspirin, indomethacin and other inhibitors of prostaglandin synthesis may be efficacious in preventing platelet aggregation in cerebral vessels.

b. An anatomical basis for understanding three-dimensional anatomico-radiological relationships of the brain and improved interpretation of cerebral angiograms is expected as a consequence of publication

this year of the monograph entitled "Computerized Tomography of the Brain - Normal Anatomy".

c. Results of recent research suggest that dilation of cerebral vessels by hypoxia or hypotension may be an oxygen-medicated phenomenon since dilation can be prevented and seizure blunted by raising local oxygen levels through the use of locally applied fluorocarbon liquid which carries a large amount of oxygen.

d. Demonstration of differential washout of stable gases such as xenon in gray and white matter of the brain at various pCO2 concentrations has been made possible by development of an improved computerized tomography technique which entailed development of a specialized mass spectrometer to measure xenon concentrations in blood and respiratory gases and a new computer program.

e. A study of innervation of cerebral blood vessels has shown that surface vessels are not more densely innervated than vessels elsewhere. However, when reserpine is used as a depleting agent, cerebral nerves were less readily depleted of norepinephrine than were peripheral nerves.

f. Findings from epidemiological studies in Honolulu and Framingham suggest that high alcohol intake is related to increased incidence of fatal and nonfatal stroke. Hypertension is still the single most important risk factor for both hemorrhagic and atherothrombotic stroke in these populations.

Significant Workshops

The Hypertension Task Force chaired by Dr. Harriet P. Dustan and Dr. Edward D. Frohlich completed a detailed report on the current state of hypertension research. The Report of the Hypertension Task Force consists of nine volumes and provides an indepth scientific reviw in the following topic areas:

Volume 1:	General Summary and Recommendations
Volume 2:	Scientific Summary and Recommendations
Volume 3:	Local and Systemic Hemodynamics
Volume 4:	Neural Control of the Circulation
	Vascular Smooth Muscle: Nerve Terminals
Volume 5:	Hypertensive Vascular Disease
	Vascular Smooth Muscle: Contractile Apparatus
Volume 6:	Pediatrics
	Genetics
Volume 7:	Prostaglandins
	Kallikrein-Kinin
Volume 8:	Renin-Angiotensin-Aldosterone
	Salt and Water
Volume 9:	Therapy
	Pregnancy
	Obesity

Major Problem Areas

Personnel Needs

The most important and urgent problem within the Division is to rebuild its professional staff to fill key vacancies resulting from departure of senior staff and building greater depth within individual programs.

Serious attention must be given not only to recruitment of the needed personnel but also to the federal policies and the environment or other reasons that have caused so many senior staff to leave. Particularly urgent is the recruitment of a new Director for the DHVD to replace Dr. Mary Jane Jesse who took a position at the University of Miami School of Medicine in April 1979.

Also leaving during the year have been:

Acting Deputy Director Dr. Jim L. Shields for a position in DEA
Chief, Hypertension & Kidney Diseases Branch for a position in NEI
Chief, Cardiac Functions Branch for a position at the University of Missouri
Scientific Project Officer for MRFIT for a position in ADAMHA
Scientist Administrator for MRFIT for a position in NIAID
Statistician, Biometrics Research Branch for a position in NIAID
Director, Framingham Heart Study retiring and accepting position at Boston University School of Medicine
Administrative Officer for a position in Health Services Administration
Statistician, Math and Applied Statistics Branch for a position at Harvard University

While all of these changes were to positions of equal or higher professional responsibility, these experienced and effective professional staff are not easily replaced.

A further indication of additional senior staff needs is the current necessity for four DHVD Associate Directors also holding dual responsibilities as Acting Chiefs of four DHVD Branches. Medical scientists with research and administrative experience are a particular current and continuing need. Policies and an environment that will attract and retain capable staff in the Division should be actively sought.

Space Needs

The DHVD space situation in the Federal Building off campus has deteriorated from its previous status of chronic inadequacy to a current status of serious deficiency that is adversely affecting the conduct of DHVD programs. The situation is made more serious by the urgent needs for recruitment of professional staff with no demonstrable space to accommodate such staff.

All offices within the space assigned to the DHVD are occupied, including the offices of the Division Director and Deputy Director even though these positions are temporarily vacant. Four Branch Chief positions are currently being filled on an active basis by the Associate Directors as dual positions. Attempting to recruit senior professional staff for such positions and for other vacant positions is impossible in the face of explanations that there is no existing office to place them in.

Space management decisions at NIH continue to be determined by formulas that do not consider physically useable space or adequacy for professional and supporting staff use. Increased demands for reporting and for increased direct program administration required readily accessible grants, contracts, and program file records and program operation materials. This is particularly needed for management of epidemiological programs, biometrics and clinical trials in which the Division is heavily involved.

Present DHVD space allocation is said to be 22,165 square feet. All reasonable actions have been taken by the Division to accommodate existing staff including dividing the office of the Director. It is essential that an expansion of space be provided so that the Division can regain adequate staffing to maintain proper administration of existing program responsibilities.

The DHVD has approved positions for 16 professional staff and 4 support staff without space to place these needed individuals.

A DHVD central journal and periodical reference room is also badly needed because of the off campus location of 90 professional staff who must keep abreast of new scientific developments to maintain high professional capabilities. Lack of such a reference room is already reflected in wasted time and excess travel required by staff to use the on campus facilities. Even worse is an increasing negative effect of this lack of convenient access to current journals and difficulty in maintaining a stimulating scientific environment for scientific productivity.

No conference room in the Federal Building is available on a regular basis or large enough to hold meetings in which key professional staff can attend. The difficulty of arranging space in Building 31 conference rooms and cumbersomeness of getting staff to the NIH Campus make frequent use of Building 31 conference rooms impractical. The DHVD has sufficient direct staff need and need for its advisory committees, policy boards and steering committees of several clinical trials to justify an adequate size conference room for regular use.

For these immediate pressing needs an additional 3,000 square feet of space in the Federal Building are needed to restore operation to adequate efficiency. These needs already have been strongly presented to the Space Management Branch and there appears to be expected approval for temporary use of 774 square feet of unoccupied space on the sixth floor of the Federal Building.

While this will provide some relief to the immediate needs for staff entering on duty in August there is urgent need for more permanent resolution of the additional space problems that must have action beginning in October.

It is imperative that the DHVD has allocated at least 3,000 square feet of space in the Federal Building to be vacated by NLM as soon as the Lister Hill Center is opened. There has been a long standing promise that the Heart Institute will be given first consideration for the NLM space on the ninth floor of the Federal Building and this must now be confirmed.

Relief from these space problems will greatly improve the potentials for recruitment of the staff needed to restore the Division to its previous strength and ability to carry out its overall mission.

DIVISION OF LUNG DISEASES

ANNUAL REPORT

October 1, 1978 through September 30, 1979

I. MISSION

The broad program goals of the Division of Lung Diseases are to achieve more effective diagnosis, treatment and, ultimately, prevention of pulmonary disease. To these ends, the Division emphasizes seven program areas: structure and function of the lung (includes lung development); chronic obstructive lung diseases (emphysema, chronic bronchitis, asthma); pediatric pulmonary diseases (neonatal respiratory distress syndrome, cystic fibrosis, bronchiolitis); fibrotic and immunologic interstitial lung diseases; pulmonary vascular diseases (pulmonary hypertension, 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 young 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. And research contracts are used to foster targeted pulmonary research and development projects.

II. PROGRESS TOWARD OBJECTIVES

In the past, a major obstacle to advances in pulmonary research was the failure of basic scientists to perceive the relevance of their disciplines to lung diseases, or to draw upon special features of the lung that could further their basic investigations. To correct this deficiency, the Division has used a variety of approaches to help basic scientists understand lung diseases and to encourage work on pulmonary problems. By including researchers from fundamental disciplines in its workshops, and by issuing requests for applications (RFAs) and for contract proposals (RFPs), the Division has reached investigators not previously concerned with diseases of the lung. The success of these endeavors is evident in the scientific accomplishments highlighted in section III of this report. Fundamental investigations supported through the Structure and Function Branch are a continuous source of knowledge that often becomes the groundwork for progress in diagnosis, treatment or prevention of specific pulmonary diseases. Such progress frequently proceeds to a point where the research becomes the responsibility of one of the Division's disease-oriented branches. Some program areas, however, continue to require stimulation through special initiatives.

The Division is strongly committed to the goal of bringing the findings of pulmonary research to bear on the practice of medicine in communities. It has taken every opportunity to bring the Division's programs to the attention of practicing physicians, other health professionals, appropriate institutions, and voluntary agencies. The *Report of the Task Force on Prevention, Control and Education in Respiratory Diseases* has been widely disseminated. Nonetheless, except for an occasional research grant application, this program area still depends on stimulation by RFPs. This year two contracts were awarded in response to a solicitation for proposals for development of a comprehensive index of an individual's risk of developing chronic obstructive lung disease. These contracts represent the initial step in a three-phase initiative directed toward disease prevention. After development and validation (phase two) of the index, the third phase will utilize the index to learn whether an individual's awareness of his or her risk of chronic obstructive lung disease, coupled with knowledge of the importance of smoking as a risk factor, will improve success rates in intervention programs aimed at cessation of smoking behavior.

Three recently initiated SCOR grants, in response to the first competition for centers addressed to respiratory failure, are examining fundamental changes in lung injury and the roles of biochemical, immunologic and hematologic alterations in relation to the progressive changes that occur in adult respiratory distress syndrome. There has long been a need for such an orderly approach to the clinical problems of acute respiratory failure, which is a frequent consequence of nonpulmonary disease and various traumas, and which is both difficult to treat and often fatal. It is anticipated that through a better understanding of the processes involved in lung injury and repair there will be progress toward earlier diagnosis of impending failure, and development of more effective ways of managing the adult respiratory distress syndrome.

Progress toward the Division's objectives has been particularly evident in the area of pulmonary vascular diseases, which now represent about 10 percent of the Division's budget, compared with 6 percent in 1975. This program area is emphasizing such fundamental problems as the role of pulmonary endothelium, as well as the development of noninvasive techniques for measurement of pulmonary hypertension and pulmonary embolism, which are techniques with important clinical applications.

Research on the fibrotic and immunologic lung diseases is focusing increasingly on the immunologic mechanisms involved in the inflammatory reactions and pulmonary fibrosis induced by a wide variety of inciting agents, and particularly by organic and inorganic dusts and gases to which workers in many occupations are exposed. Since at present avoidance of exposure is the only preventive measure, these fundamental studies offer a promising approach to development of interventions that will prevent or arrest the progressive lung damage that characterizes this group of diseases.

With the dramatic progress that has been made in reducing mortality from neonatal respiratory distress syndrome, there has been a surge of interest in bronchopulmonary dysplasia, a disorder of increasing incidence because

more infants with RDS are surviving. A workshop has been organized to address this problem and to suggest research approaches for dealing with it.

With regard to emphysema and chronic bronchitis, the success in elucidating the role of protease-antiprotease interactions has been such that researchers are now turning to the possibility of developing synthetic inhibitors that may be used for replacement therapy in persons genetically deficient in alpha-1-antitrypsin. At the same time, basic investigations are moving into new areas of exploration that involve other antiproteases (such as alpha-2-macroglobulin) and other agents (such as free radical oxidants) that may have a role in development of emphysema in persons who are not genetically deficient in alpha-1-antitrypsin.

The Division has long recognized the importance of epidemiologic studies to furtherance of its goals. But for several reasons this facet of the lung program has been slow to develop. Some obstacles--the cost and long-term commitment required--are inherent in the epidemiologic approach. These difficulties have been coupled with the absence of a well-defined plan for a lung program based on an indepth analysis of the state of knowledge and unmet needs in this area of investigation. A group of experts has been addressing this problem since May 1978, when the 13-member Task Force on Epidemiology of Respiratory Diseases was constituted. More than 60 epidemiologists, biomedical researchers or pulmonary physicians have participated in this undertaking. The penultimate meeting of the task force was held at the end of July 1979, and the final report should be ready for distribution early in 1980. There is every reason to expect this report to provide an impetus to the Division's epidemiology program, similar to the stimulus the reports of the Task Force on Research in Respiratory Diseases, and of the Task Force on Prevention, Control and Education in Respiratory Diseases, have given other facets of the lung program.

To ensure programs of high quality, the Division continues its periodic assessment of all activities that result from its special initiatives. This year the 19 SCOR programs that were awarded about 18 months earlier were visited by expert consultants and staff to assess progress at each center. The reviewers were asked to determine whether the centers were meeting the requirements for a SCOR as outlined in the Division's announcement of the competition, and whether they were progressing toward fulfilling the scientific goals stated in the grantee's application. The site visit report was sent to the SCOR director for his or her guidance in planning the future direction of the center's program. With few exceptions, the SCORs were progressing well. But in several instances the visits served to identify weaknesses that could be corrected to strengthen the program at a given center. The reports were only for the information of the SCOR director and the Division staff, and to provide the Pulmonary Diseases Advisory Committee with an overall evaluation of the status of the SCOR program.

The contract program is regularly monitored by asking outside experts to determine the extent to which a group of completed contracts, which were awarded in response to a particular RFP, has fulfilled the objectives stated in the Division's solicitation. This year the Division issued three such evaluative reports, and a fourth is in preparation. These reports serve not

only to inform the Division staff, the Division's Advisory Committee, the NHLBI Council and the biomedical community what has (or has not) been accomplished, but they also point to areas of investigation that need further attention.

This year marked the end of a decade for the lung program, a fact that was recognized by the American Thoracic Society and American Lung Association at a dinner for the Division of Lung Diseases that was held in Las Vegas in association with the annual ALA-ATS meeting. Dr. Julius Comroe and Dr. Theodore Cooper described the changes in pulmonary research and medicine that have resulted through the Division's activities, and Dr. Robert Levy spoke for the Institute. An editorial in the July 1979 issue of the American Review of Respiratory Diseases highlights the status of the Institute's lung program in 1969 and what has transpired during the ensuing years.

III. HIGHLIGHTS OF ACCOMPLISHMENTS

In fairness to the many investigators who are contributing to the understanding and management of pulmonary diseases, it should be stated that the accomplishments reported here do scant justice to numerous advances during the past year. Accomplishments identified in this section are based on annual reports of grantees and contractors, annual summaries by SCOR and program project directors, and letters from investigators who respond to a biennial request for a summary of their major scientific achievements during the past two years.

Investigations of fundamental mechanisms, undertaken because of their theoretical interest or in relation to a particular disease process, are often found to contribute insights into the etiology or pathogenesis of various pulmonary disorders. A noteworthy example is the confluence of information about cellular antioxidant systems, which is emerging from investigations pursued in several laboratories in different contexts. The effects of nonphysiologic levels of oxygen on the enzyme systems of cells are important in relation to the treatment of neonatal and adult respiratory distress syndrome, chronic bronchitis and emphysema, all of which are characterized by hypoxia and for which oxygen administration is a standard mode of therapy.

The toxic effects of excessive exposure or high levels of oxygen, which is often a complication of such therapy, has been shown to be associated at the cellular level with the presence of toxic free radicals of oxygen. Recent studies have shown that the enzyme superoxide dismutase has a protective role, converting the free radicals into nontoxic products. It has now been found that when animals are treated with this enzyme before exposure to high levels of oxygen it prevents one of the changes (decrease in serotonin clearance by the lung) that is an early indicator of lung damage. Moreover, when neonatal animals are exposed to almost pure oxygen, superoxide dismutase activity increases, but this does not occur when adult animals are similarly exposed. This observation is consistent with the fact that immature animals are more resistant than the adult to oxygen toxicity. Another observation, and one of potential therapeutic value, is that a single dose of endotoxin protects animals from lethal hyperoxic

lung damage. Moreover, a month after recovery there is only slight diffuse lung damage in the treated animals, whereas in animals not treated with endotoxin there was either death or marked pulmonary fibrosis after recovery. It is believed that endotoxin activates the lung antioxidant system, a supposition based on the fact that activities of both superoxide dismutase and other antioxidant enzyme were increased after the endotoxin exposure.

Other recent fundamental studies have linked oxidants in cigarette smoke to inactivation of alpha-1-antitrypsin, the protease inhibitor that prevents degradation of lung elastin by the protease elastase. In test tube experiments it has been shown that addition of antioxidants prevents the inactivation of alpha-1-antitrypsin. If confirmed in animal and human studies that are now in progress, this finding is of theoretical interest because it shows for the first time a possible mechanism to account for the strong association between cigarette smoking and occurrence of emphysema. But it is also of potential clinical value as it may be possible to block the adverse oxidizing effects of cigarette smoke by means of appropriate pharmacologic agents that prevent inactivation of alpha-1-antitrypsin.

Another example of mutually reinforcing basic and disease-related research is the rapidly expanding investigative effort addressed to immunologic processes. It is increasingly evident that immunologic responses are involved not only in diseases like asthma and hypersensitivity pneumonitis, in which antigens are the inciting agents, but also in adult respiratory distress syndrome, fibrotic diseases that result from inhalation of inorganic dusts, pediatric pulmonary diseases such as cystic fibrosis and bronchiolitis, and in exacerbations of chronic bronchitis and emphysema that result from respiratory infections.

Receiving considerable research emphasis is the role of the serum complement system in inflammatory reactions of the lung. Activated complement components C3 and C5 are of particular interest because they are chemotactic agents responsible for leukocyte migration to sites of tissue injury, stimulate mast cells and basophils to release histamine, increase phagocytosis by polymorphonuclear leukocytes, and increase leukocyte adherence. These activated complement components may result when complement is fixed by antigen-antibody complexes, but complement activation may also result from alternate metabolic pathways stimulated by other agents. Recent studies of the effect of complement C3 on neutrophil adherence support the hypothesis that activated complement contributes to lung damage by stimulating aggregation of leukocytes, which are then sequestered in pulmonary capillaries where they release lysosomal enzymes. Other studies, designed to identify the common denominator in adult respiratory distress syndrome resulting from shock and sepsis, also suggest that the complement system is a mediator of lung injury. It is believed that the lysosomal enzymes released by leukocytes damage the endothelial cells, thus altering their permeability and contributing to the pulmonary edema that occurs in this syndrome. Complement C5a has been found in lavage specimens of bronchoalveolar secretions, and quantitative studies are in progress to determine whether the levels of complement increase in diffuse lung injury.

In addition to advances in elucidation of basic mechanisms, noteworthy progress is being made in development of tests that can measure subtle changes in the static and dynamic properties of the lung, and are now being studied in various disease states. At the same time, there is considerable interest in, and progress toward, development of techniques that meet special needs. For example, to monitor changes in the course of therapy of critically ill patients, tests must not only provide accurate assessments but must be minimally invasive, or noninvasive. For epidemiologic studies, tests of pulmonary function must be suitable for use on large numbers of persons who must be screened with minimal inconvenience and loss of time to the subjects, and should involve minimal cost to the study. For children who are too young to cooperate in the performance of pulmonary function tests, the techniques must be accurate, able to be used rapidly, and preferably noninvasive.

Among recent advances toward these various ends are radiographic techniques suitable for epidemiologic surveys, a noninvasive method of measuring pulmonary function in infants and young children, and a minimally invasive method of assessing damage to pulmonary capillaries in disease states. A very promising technique for use in young children introduces oscillating airwaves at the mouth and measures their reflection as they traverse the tracheobronchial tree. It provides information about the geometry of the airway and identifies airway obstruction. The problem of a suitable device that connects the apparatus to the mouth and introduces the airwave has now been solved, and the system provides a simple safe procedure that permits frequent and repeated assessment of an important aspect of lung function. Advances are also being made in development of radiographic techniques that permit rapid, relatively inexpensive assessments of lung function suitable for epidemiologic surveys. Computerized calculation is used to measure total lung volume from chest radiographs. And radiographic techniques have now been improved to the point that they can visualize the smallest airways by computer enhancement of the image and magnification. Moreover, the technique has been tested in living animals and shown to be usable while the lung is moving. Another promising technique that is minimally invasive, and should be suitable for use of very ill patients, delivers by aerosol a hydrophilic radionuclide of low molecular weight. Regional disappearance from the lung, measured by gamma camera, is influenced by the permeability of the alveolar-capillary barrier. The method has been shown to identify increased permeability in patients with interstitial lung diseases and is now being tested in patients with pulmonary edema and adult respiratory distress syndrome. It is expected to make it possible to distinguish between edema due to increased permeability and due to increased vascular hydrostatic pressure.

IV. REPORTS OF WORKSHOPS, MEETINGS, OTHER

A. Workshop Reports

- Report of Workshop on Defense Mechanisms of Lung Parenchyma
  NIH Publication No. 79-1669

B. Evaluative Reports

- Evaluative Report on Completed Contracts Awarded in Response to RFP NHLI 74-15: An Educational Program for Early Treatment of Acute Respiratory Insufficiency
  DHEW Publication No. (NIH) 79-1652

- Evaluative Report on Completed Contracts Awarded in Response to RFP NHLI 73-21: Studies of Normal Lung Cell Separation, Culture and Morphology
  DHEW Publication No. (NIH) 79-1657

- Evaluative Report on Completed Contracts Awarded in Response to RFP NHLI 75-5: Identification of Lung Cells
  DHEW Publication No. (NIH) 79-1664

- Evaluative Report on Completed Contracts Awarded in Response to RFP NHLI 74-7: Development of an In Vitro Diagnostic Test for Sarcoidosis
  NIH Publication No. 79-1668

C. Other Reports

- Report of Task Force on Bilateral Carotid Body Resection
  DHEW Publication No. (NIH) 79-1416

- Report to National Heart, Lung, and Blood Advisory Council
  November 20-21, 1978 (limited distribution)

- Analysis of Pulmonary Research Programs Supported by NHLBI in Fiscal 1978 (limited distribution)

- National Heart, Lung, and Blood Institute, Division of Lung Diseases Program Report: Fiscal 1978
  DHEW Publication No. (NIH) 79-1659

V. MAJOR PROBLEM AREAS

The problems identified below are the same as those reported last year. The situation has not changed in the interim since the 1978 report.

A. Funds for a Prevention and Control Program

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.

B. Funds for New Initiatives

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 has markedly increased within the past two years, it is now 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.

ANNUAL REPORT

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

October 1, 1978 Through September 30, 1979

Through its programs, 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. These programs deal with four major areas: (1) Thrombosis and Hemostasis, (2) Red Blood Cell Disorders, (3) Blood Resources, and (4) Sickle Cell Disease. 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, 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 federal and nonfederal 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 selected highlights of fiscal year 1979 progress toward the attainment of these objectives are summarized in the following paragraphs.

THROMBOSIS AND HEMOSTASIS

The long-range objective of this program is to develop new knowledge which could 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

Aspirin is known to affect the clotting system by inhibiting the aggregation platelets through the inactivation of an enzyme (cyclooxygenase) which is involved in the production of a potent aggregating agent, thromboxane A_2. It h been shown that half an aspirin tablet a day is effective in almost totally eliminating platelet cyclooxgenase activity. However, the endothelial cells lining the blood vessels also have a cyclooxygenase system, but it leads to t production of a potent vasodilating and platelet disaggregating substance, pr stacyclin. In tissue culture experiments, the endothelial cell cyclooxygenas system has been shown to be far less sensitive to aspirin than the platelet system. Therefore, clinical studies have been undertaken to test the hypothe that aspirin dosages can be established which will inactivate the platelet ag gregating system and which will have no significant effect on the protective system in the vessel wall.

Fibronectin, a major protein of vertebrate blood and cell surfaces, may be th "scaffolding" for collagen. The chemical crosslinking of collagen-fibronecti mediated by the coagulation enzyme Factor XIIIa, may be the mechanism for insolubilization of fibronectin in tissues and cell matrices. Factor XIII defi ciency, therefore, may be a cause of poor wound healing and excessive scarrin resulting from the failure of the fibronectin-collagen interaction to occur. Further, fibronectin levels have shown to decrease with sepsis and other diseases. This depletion may contribute to organ failure in traumatized and other severely ill patients. Cryoprecipitate, a blood plasma fraction rich i fibronectin, may offer a new approach to treatment and prevention of multiple organ failure.

Plasma samples can be obtained in the mid-trimester from fetuses at risk for severe hemophilia A. A radioimmunoassay for factor VIII coagulant activity, VIII: C(Ag), now permits the determination of the presence or absence of hemophilia. With this information, an informed decision can be made concerning therapeutic abortion. In addition to currently available genetic counseling, this tool may have future applicability to the diagnosis of hemophilia B and other hemorrhagic disorders.

RED BLOOD CELL DISORDERS (EXCLUSIVE OF SICKLE CELL ANEMIA)

This program is concerned with the development of new knowledge in the areas of Cooley's Anemia, aplastic and refractory anemias, as well as hemolytic anemias. 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 which can cure or prevent the 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 erythropoiesis for research and clinical use. (3) Red Blood Cell Membrane 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 status of patients afflicted with various hemolytic anemias.

Highlights

Measuring two minor hemoglobin (Hb) fractions, HbA_{1c} and $HBAl_{a+b}$, has value in monitoring the control of plasma glucose in patients with diabetes. Because of a chemical reaction between sugar and normal hemoglobin, hyperglycemia causes an increase in HbA_{1c}. Unlike single measurement of plasma glucose or 24-hour urine glucose, this new measurement technique provides an assessment of the degree of hyperglycemia over the life span of the red blood cell. The micromethod is accurate and can be used for a large number of samples; in addition, HbA_{1c} can be measured by immunologic methods. Not only can these tests be used to monitor the control of diabetes, but they are also useful in studying other proteins which may play a role in the pathogenesis of diabetes.

A radioimmunoassay (RIA) for erythropoietin, one of the prime regulators of red blood cell production, has recently been reported. This RIA appears to offer significant advantages over exising bioassays. The RIA is sensitive to an amount of erythropoietin approximately one hundred times smaller than the amount required for bioassay. With projected improvements and validation, this assay appears to offer promise of being a simple, reliable assay useful both as a diagnostic aid and research tool.

BLOOD RESOURCES

The mission in blood resources is directly related to the National Blood Policy goal of an adequate supply of high quality blood and blood products. In pursuit of this goal, the blood resources program supports activities to improve 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 five 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 national 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 for use as blood substitutes in transfusion therapy, organ perfusion and other promising applications. (4) Blood Component Therapy: Develop definitive guidelines for the clinical use of 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. (5) Transplantation Biology: Support fundamental research in immunology, immunogenetics, and other aspects of transplantat biology.

Highlights

A completely closed, continuous-flow plasmapheresis system has been develope This system, a form of automated plasmapheresis which employs microporous mem brane filtration technology, will probably be the most significant technologi cal development in blood banking over the next few years. Because it reduces plasma collection time from the current 1-1/2 hours to 30 minutes, it is predicted that the number of volunteer donors will increase significantly. Further, the microporous membrane filtration system is completely closed and thereby enhances both donor and recipient safety.

The National Blood Data Center, supported by an NHLBI contract, reported its first national estimates on blood resources. Highlights of these data includ the following. In the period from 1971 to 1978, whole blood transfusions decreased from 67% to 21% of all transfusions while red cell transfusions increased from 19% to 48% during the same time period. In that same time perio both platelet concentrate and single donor plasma transfusion rates more than tripled. All four of these trends are important because they indicate that transfusion practices recommended by the NHLBI are increasingly being followe

SICKLE CELL DISEASE

The mission of this program is to reduce morbidity and mortality due to sickl cell disease. The following objectives have been established: continue basi research into the pathophysiology of the disease process at the molecular, cellular, and clinical levels; develop improved methods of clinical care; develop a more rational approach to patient management based on the latest scientific advances; provide accurate, up-to-date information to health care providers and consumers; and evaluate the effectiveness of education, screeni and counseling programs.

Highlights

Among the various strategies proposed to inhibit intracellular hemoglobin S polymerization in sickle cell disease, the most exciting and straightforward is the use of small molecules that can bind the regions of the hemoglobin S molecular surface involved in an intermolecular bond in the polymer. An essential requirement to designing stereospecific inhibitors is a detailed knowl edge of the regions of the hemoglobin S molecular surface that are involved in intermolecular bonding. Significant advances have been made and much information about these regions has recently become available. It now appears that the double strand of molecules found in the deoxyhemoglobin S single crys tal lattice is also a basic element of the polymer structure that occurs inside sickled red blood cells. A recent study has shown that X-ray diffractio

patterns of fibers of deoxygenated sickle hemoglobin and of the needles that appear as replacements for normal fibers are strikingly similar to the rotation patterns of diagrams of single crystals grown according to earlier methods. The similarity suggests that the crystal structure can serve as a model of fiber, although some differences between fibrous and crystalline patterns exist. The crystal structure consists of two molecules in the asymmetric unit that are related to each other by a noncrystallographic a-axis. These form a pair of filaments, each of which is translated with respect to its neighbor. The contact regions in the crystal between filaments involve the residue at the mutation site, valine β 6, on one molecule and hydrophobic residues on the β -chains of the neighboring molecule in the other filament of the pair. The stabilization of the fiber is probably dependent upon the same hydrophobic interactions that occur in the crystal. The pairing of filaments and their aggregation could be responsible for the sickle of SS erythrocytes.

Professional Training and Development

As a focus of blood research training, the Institute cooperates with other NIH Institutes, professional societies, and with other Governmental agenices to carry out a continuing assessment of the national personnel needs for research in blood diseases and blood resources. To assist in meeting these needs, the Institute currently supports 171 trainees in blood diseases and resources.

Workshops

A. A consensus development conference entitled "Hypertransfusion Therapy for Sickle Cell Disease Patients" was held on April 23-24, 1979, in Bethesda, Maryland. A summary will appear in the Annals of Internal Medicine.

B. "Workshop on Therapeutic Plasma Cytapheresis," co-sponsored by the Mayo Clinic/Mayo Foundation, was held on April 25-26, 1979 in Rochester, Minnesota. Proceedings are in preparation for publication.

C. Workshop/conference on "Aplastic Anemia - A Stem Cell Disease," co-sponsored by NIAMDD, DRG, and the FIC was held in San Francisco, California on June 17-19, 1979. A summary and highlights will be published in the October 1979 issue of Blood.

D. "1979 Conference on the Management and Logistics of Blood Banking," co-sponsored by the American Blood Commission, was held on June 21-22, 1979, in St. Petersburg, Florida. Conference proceedings will be published by the NHLBI.

E. Conference entitled "Second International Symposium on the Red Blood Cell and the Lens Metabolism," co-sponsored by the NEI, NIAMDD, FIC, and the University of Texas, will be held on October 26-28, 1979, in Galveston, Texas.

NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
DIVISION OF EXTRAMURAL AFFAIRS
Annual Report
October 1, 1978 - September 30, 1979

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 processing services for the Institute, (b) initial scientific and technical merit review of certain competing grants and all research contracts for the Institute, and (c) committee management functions for the entire Institute.

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 formulation of 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 1979 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 grant, 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. Preparation of review materials for Council, staff, and Institute initial review groups.

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

7. Operation of the Program Policy and Procedures Office.

8. Committee management functions.

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 1979, the types of reviews in the grant program included:

Pulmonary Academic Awards
Medical School Pulmonary Faculty Training Awards
Preventive Cardiology Academic Awards
National Research Service Awards for Institutional Training Grants
Special Emphasis Research Career Award in Diabetes
Minority Hypertension Research Development Summer Program
Program Project Grants
Specialized Centers of Research on Ischemic Heart Disease
Hypertension, Ischemic Heart Disease, Pulmonary, Arteriosclerosis, and Thrombosis SCOR Supplemental Grants
National Research and Demonstration Center Program (Supplement)
Comprehensive Sickle Cell Center Supplemental Grants
Specialized Centers of Research (SCOR): Hypertension
National Research and Demonstration Center Program (Supplement)
Clinical Trials Grants
Cooperative Clinical Research Grants
Research Demonstration and Dissemination Project Grants
Education Project Grants
Conference Grants
Research Projects Grants
Pilot Evaluation Studies of Community High Blood Pressure Control in Two Rural Communities with High Prevalence of Hypertension (RFA)
Blood Material Interactions (RFA)
Interdisciplinary Studies on the Role of the Central Nervous System in Hypertension and Control of Normal Blood Pressure (RFA)
Studies on the Control and Differentiation of Mucous Producing Cells (RFA)
The Effect of Diet on the Metabolism and Blood Levels of High Density Lipoproteins and Other Lipoproteins (RFA)
Isolation and Characterization of Plasma Proteins With New Therapeutic Uses (RFA)
Bronchial Secretions: Physical and Chemical Studies (RFA)
Response of the Pulmonary Endothelium to Injury (RFA)
Studies on the Effects of Hypertension and Vasoactive Agents on the Vasculature (RFA)
The Development of an Intravascular System for Assaying Characteristics of the Sickle Erythrocytes (RFA)

In addition, numerous new, renewal, and unsolicited contract proposals were reviewed.

The Review Processing Section continued to expand the number of diverse functions it performs. In addition to receiving, recording and distributing all grant applications and contract proposals reviewed by the Review Branch, this Section coordinated paper flow with review schedules; handled all administrative details before, during, and after all committee meetings; collated, distributed and mailed review materials to consultants and Institute staff involved in project site visits, and grant and contract review committee meetings; maintained the master calendar for the entire branch and issued the review schedule. This Section also initiated a computer program for inputting the data for the yearly workload report; instituted the issuance of the "Grant and Contract Log" to the review branch staff; and assumed the responsibility of the "Review Branch Consultant Contact List". During RFA, training, and SCOR committee meetings, processing staff prepared the budgets for every approved application reviewed. It maintained, managed and computerized the consultant file system; made all travel arrangements and prepared vouchers for all government employee consultants; and maintained effective liaison with all Review Branch Sections, other DEA Branches, NHLBI program divisions, the Division of Research Grants, other BID's and several central NIH offices.

The Grants Operations Branch was responsible for approximately 3,300 awards, with a dollar value in excess of $350,000,000 during Fiscal Year 1979. The Branch's responsibilities included the formal issuance of awards as well as the day-to-day fiscal and administrative management of those awards. The Branch was also actively involved in site visits, Council preparation and grant budget negotiations, as well as serving as an important interdivisional coordinator. Senior representatives of the Branch continued to be most effective in assisting NIH-OD staff in the development and analysis of NIH grant policy and procedure. The Branch's continued efforts to improve its management practices and procedures have undoubtedly resulted in dollar dollar savings for the Institute.

The Processing Section of the Grants Operations Branch continued to improve the maintenance of grant files (pending, active, terminated) and continues to initiate and check all encumbrance lists. The Section also received, reviewed, and filed or appropriately forwarded items pertaining to NHLBI grants and awards (e.g., activation notices, appointment forms, termination notices, payback forms). The Processing Section has proposed, developed and instituted the NHLBI's first experiment with flexitime; so far, this has been a resounding success. With the increasing workload relative to the preparation of Council Books the associated problems have multiplied: (1) Multipage summary statements must be properly prepared by hand for the books to bind properly. (2) A majority of DBP code assignments do not get recorded before summary statements are prepared for the Study Section. Thus this code is not on summary statements when received and must be obtained before Council Books can be collated. (3) At least 10% and more of the duplicated summary statements have not been received by the date of mailing for Council Books. Many of these must be duplicated by staff.

Supplemental material prepared by program staff (RFA's, program plans, staff Summary Statements, etc.) are not or cannot be supplied in sufficient quantity so these must also be duplicated.

The Contracts Operations Branch, responsible for the administrative aspects of the NHLBI contracts program, continues to be involved in a variety of activities: presolicitation, 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 1979, Secretary Califano has continued a major survey of the DHEW grants and contracts program, and ordered a number of significant moves to correct deficiencies in these programs. As a result, fewer noncompetitive contracts are awarded by DHEW components and a major shift in the distribution of award dates will occur. To more evenly distribute workload throughout the fiscal year, an effort has been mounted to reduce the number of awards in the fourth quarter of the fiscal year.

In conjunction with Secretary Califano's initiatives, a program was begun to certify all contracting officers and contract specialists. To date, eight NHLBI contracts specialists have been certified.

The contracts program was comprised of approximately 450 contracts and reimbursable agreements, representing approximately $85,000,000 during FY 1979. The Branch staff continued to work closely and effectively with the program staff but with the appropriate and necessary independence that denotes professionalism.

The Reports and Evaluation Branch was reorganized and relocated within the administrative structure of the Institute. It is now the Information Systems Branch, Office of Program Planning and Evaluation, Office of the Director. Because the information systems of the Institute require a large input from DEA for update and corrections of records, we have continued to maintain a close liaison with this branch. Also we utilize a considerable output from the branch in the operation of review committees and the Council.

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 Processing Section of the Grants Operations Branch, the workload relating to Council preparation has continued to grow so that the space alloted for that purpose is now quite cramped. In the Contracts Operations Branch, the accommodations for staff assigned to DHVD are dark, crowded, and barely suitable.

The staff has continued to perform in excellent fashion and morale appears to be generally good within the Division. Staff turnover has exceeded the expected level but we have managed to fill most vacancies. Additional positions for understaffed areas, especially processing units, are urgently needed to insure maximum efficiency and productivity.

INTRAMURAL RESEARCH

THE NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

ANNUAL REPORT

October 1, 1978 - September 30, 1979

INTRAMURAL RESEARCH

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ANNUAL REPORT
LABORATORY OF BIOCHEMISTRY
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

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A. Regulation of Enzyme Levels

(a) Specific Enzyme Degradation

The differential proteolytic degradation of proteins in vivo serves two functions: (1) It is a means by which defective gene products and other imperfect nonfunctional proteins are eliminated. (2) It operates in concert with mechanisms of protein synthesis to regulate the intracellular level of normal enzymes. Further progress in understanding the mechanism of normal enzyme breakdown and factors that regulate it are contingent upon the development of cell-free enzyme systems that can catalyze the preferential degradation of those enzymes shown to undergo degradation in vivo.

(i) Glutamine Synthetase. The inactivation of glutamine synthetase in extracts of Klebsiella aerogenes is believed to represent the first step in the degradation of this enzyme during nitrogen starvation in vivo. Many characteristics of the inactivation reaction (see 1978 Annual Report) are similar to those of the cytochrome P-450-dependent mixed function oxidation reactions, catalyzed by mammalian microsomal preparations. Because ascorbate can mimic in many respects the microsomal system, studies on the nonenzymic ascorbate mediated inactivation of glutamine synthetase have been undertaken to develop a model system that might help to elucidate the mechanisms of the more complex enzyme system. As with the enzyme system, ascorbate induced inactivation requires O_2, is stimulated by Fe^{3+}, and is inhibited by chelating agents, Mn^{2+}, catalase, and horseradish peroxidate; also, addition of superoxide dismutase or of free radical scavengers (ethanol and BHT) were without effect. In the absence of metabolic effectors, ascorbate-dependent inactivation occurred with both adenylylated and unadenylylated glutamine synthetase; however, in the presence of both glutamate and MgATP, inactivation of unadenylylated enzyme was inhibited, whereas inactivation of the adenylylated enzyme was stimulated. This suggests that the ascorbate-induced inactivation reaction may mimic a physiologically important mechanism of cellular regulation in which the inactivation reaction may represent an initial step in the normal proteolytic degradation process. This hypothesis is supported by preliminary experiments showing that extracts of catalase-deficient strains of Escherichia coli exhibit greater proteolytic activity than do extracts of cells, and also by the observation that the ascorbate inactivated glutamine synthetase is degraded more rapidly than native enzyme when incubated with cell-free extracts of catalase-deficient strains of E. coli.

(ii) Glutamate Dehydrogenase. The observation that purified preparations of yeast protease B could degrade the NADP-dependent glutamate dehydrogenase (NADP-GDH) in vitro, suggested that this protease is implicated in the degradation of NADP-GDH which occurs when Saccharomyces cerevisiae is exposed

to carbon starvation (see 1978 Annual Report). This proposal is contraindicated by studies showing that the rates of NADP-GDH degradation in carbon starved cultures of a mutant strain lacking protease B (obtained from E. W. Jones, Carnegie Mellon University) is the same as that in wild type strains. Similarly, the rate of GDH degradation is unaffected by mutations that led to low intracellular levels of proteases A, B, and carboxypeptidase y. Thus, either the lysomal proteases of yeast are not implicated in the selective degradation of normal enzymes, or normal enzyme degradation involves another as yet unidentified protease in these organelles.

(iii) Effect of Carbon on Nitrogen Starvation. The regulatory function of enzyme degradation in metabolism is indicated by previous studies in this laboratory showing that the levels of 12 different enzymes are differentially affected when E. coli is subjected to conditions of nitrogen or carbon starvation. Further studies show that during nitrogen starvation, the activities of glutamate dehydrogenase (GDH), deoxyarabino-heptulosonate-phosphate synthase, ornithine transcarbamylase (OTCase) and aspartokinase-III (AK III), decline more rapidly (> 10% per hour) than the reported rates of general protein turnover (3-4% per hour); the activities of malate dehydrogenase, aspartokinase-I, glutamate synthase, and aspartate transcarbamylase declined at rates of 4-8% per hour; whereas the activities of α-ketoglutarate dehydrogenase, phosphofructokinase (PFK), aldolase, malic enzyme, phosphoenolpyruvate synthase, glutamine synthetase, and glucose-6-P dehydrogenase (G6PDH), and 6-phosphogluconate dehydrogenase (6PGDH) either remained constant or increased in activity. During glucose starvation, the activities of GDH, ATCase, OTCase, AK III, and glutamate synthase declined, but less rapidly than during nitrogen starvation; whereas glutamine synthetase, PFK, aldolase, G6PDH, and 6PGDH remained constant or increased slightly. Since addition of uracil (to repress synthesis of ATCase) or of lysine (to repress synthesis of AK III) had little effect on the rates of inactivation of these respective enzymes, it appears unlikely that resynthesis of these enzymes takes place during starvation.

From the inhibitory effects of chloroamphenicol on the inactivation of GDH and AK III during nitrogen starvation, and on the restoration of these activities when ammonia is added to nitrogen cells, it is concluded that the loss of these enzyme activities is most likely due to enzyme degradation. In efforts to optimize the conditions for inactivation of GDH and AK III, it was found that inactivation due to nitrogen starvation was the same whether glycerol, glucose, succinate, gluconate, or acetate was the carbon source. However, among 6 different strains of E. coli examined, the rate of inactivation varied from < 1% to 40% per hour.

(b) Enzyme Synthesis

Genetic and biochemical studies with enterobacteria, which have been carried out in several laboratories, indicate that a common mechanism underlies regulation of the synthesis of several enzymes involved in nitrogen metabolism, and that glutamine synthetase might play a key role in this regulation. A glutamate carbon utilizing strain of E. coli, N99G⁻, was

isolated as a spontaneous mutant of E. coli K12 strain N99. Unlike the parental strain, N99G-1 can utilize glutamate as a carbon source in the presence of excess ammonia. Also, with N99G-1, the patterns of repression and derepression for glutamine synthetase, glutamate dehydrogenase, and glutamate synthase which are elicited by variations of the composition of the growth media, are significantly different from the patterns observed with the parental strain. The possibility that the Glu^+ mutation may reflect a fundamental regulatory alteration is suggested by the discovery that the Glu^+ mutation is accompanied by simultaneous loss in sensitivity of the cell to lysis by the transducing phage, P1; moreover, the fact that spontaneous Glu^- revertants of N99G-1 are sensitive of P1 lysis indicated that the $gluC^+$ mutation is pleiotropic.

B. Regulation of Enzyme Activity

(a) Regulation of Covalent Modification

(i) Cascade Theory. A previous theoretical analysis of cyclic cascade systems showed that they are endowed with an exceptional regulatory characteristic, especially with respect to metabolite controls. A further analysis of time-dependent functions shows that multicyclic cascades serve also as rate amplifiers with respect to the modification and demodification of the interconvertible enzyme in the cascade. Modification of this enzyme proceeds with a lag whose duration increases as the number of cycles in the cascade increases. The initial lag is followed by a burst of interconvertible enzyme modification. The steepness of this burst increases enormously as the number of cycles in the cascade increases. This rapid acceleration in rate derives from the fact that the rate of covalent modification of the last interconvertible enzyme in the cascade is a multiplicative function of the rate constants of all the reactions that lead to modification of the enzyme. The magnitude of the amplification can vary enormously, depending on the number of cycles, values of the kinetic constants, and the concentrations of metabolite effectors and of the cascade enzymes. These studies demonstrate that interconvertible enzyme cascades can respond to allosteric stimuli within the millisecond time range.

(ii) Construction of an Experimental Monocyclic Cascade Model. An artificial in vitro enzyme system has been developed to investigate the regulatory characteristics of a monocyclic interconvertible enzyme cascade. The system consists of: (a) histone 1, which was purified from rabbit thymus, to serve as the interconvertible protein substrate; (b) a cyclic AMP-dependent protein kinase which has been purified to near homogeniety from bovine heart muscle; and (c) a phosphoprotein phosphatase, also purified from bovine heart. From a detailed kinetic and steady-state analysis of this well defined in vitro system, and studies on its regulation by cyclic-AMP, Pi, and other allosteric effectors, it will be possible to test the validity of the steady-state functions which have been derived from a theoretical analysis of a monocyclic cascade model.

3

(iii) Escherichia coli Glutamine Synthetase.

(1) Studies with permeabilized cells. After freezing and thawing, and exposure to the nonionic detergent, Luberol, E. coli cells become permeable to small molecules, but retain their intracellular enzymes. Glutamine synthetase and converter enzymes of the cascade system that control its activity are fully active in the permeabilized cells, and their activities can be measured in situ by incubations in the same assay mixtures used to measure their activities in extracts. Preliminary studies indicate that the adenylylation and deadenylylation of glutamine synthetase in permeabilized cells is regulated by the ratio of α-ketoglutarate and glutamine, as was predicted from experiments with cell-free extract. A systematic analysis of permeabilized cell preparations is now in progress to determine if unique features of cascade control, which have been disclosed by theoretical considerations and by studies with isolated enzymic preparations, are demonstrable in intact cells.

(2) Enrichment of converter enzymic levels by genetic manipulation. By means of chemical mutagenesis, two E. coli mutants have been obtained with defects in the GlnD gene which encodes for the uridylyltransferase-uridylyl removing enzyme(s). These strains are unable to make the uridylylated form of the P_{II} regulatory protein, and are therefore unable to catalyze the deadenylylation of glutamine synthetase. Consequently, they require glutamine for growth because the glutamine synthetase they produce is all in the adenylylated (catalytically inactive) form. By screening a collection of 2,000 E. coli strains carrying ColE1 plasmids (Clarke and Carbon, 1976), two hybrid plasmids-containing strains were found that could complement the GlnD mutations. These strains produce 14 to 25 times as much uridylyltransferase-uridylyl-removing enzyme(s) as does the wild type strain, and will therefore be used for the purification of this (these) enzyme(s).

(3) Use of Cibacron Blue to monitor structural alterations of glutamine synthetase structure. The binding of Cibacron Blue to the nucleotide binding site of kinases and dehydrogenases elicits a shift in the spectrum of the dye which can be measured by difference spectroscopy. Earlier studies showed that a different spectral perturbation is provoked by binding of the dye to the taut and relaxed forms of the dodecameric enzyme, as well as to dissociated subunits. In the meantime, it has been found that commercial preparations of Cibacron F_3GA are composed of as least 8 different molecular species. The four major components have been purified to near homogeniety by silica gel column chromatography. Whereas all four species have a similar if not identical absorption spectra, and all can react at the nucleotide binding site of glutamine synthetase ($Kd \simeq 10^{-7}$ M), the difference spectrum obtained by binding to a given enzyme form is different for each dye component. The spectral perturbation associated with the primary interaction of one dye component (fraction 1) with the taut form of glutamine synthetase is followed by a time-dependent shift in the defference spectrum which is accompanied by the inability of the complex to be dissociated by high concentrations of ADP. These preliminary studies indicated that the various dye

species can serve as reporter groups to monitor changes in protein conformation associated with the relaxation and subunit dissociation reactions, and possibly also with the binding of substrates and allosteric effectors to the enzyme.

(4) Reaction of adenylylated subunits with anti-AMP antibodies. From measurements of the rapid quenching of tryptophan fluorescence which occurs when anti-AMP antibodies react with adenylylated subunits, it is evident that the primary binding of antibodies is independent of the state of adenylylation of GS; however, the capacity of these interactions to yield macromolecular lattices is dependent on both the number of adenylylated subunits per molecule and the total concentration of adenylylated subunits. Dissociation and resasociation of mixtures of fully adenylylated and unadenylylated GS preparations leads to the production of hybrid molecules which exhibit immunoprecipitation patterns with anti-AMP antibodies that are similar to the patterns obtained with naturally occurring GS hybrids. Preliminary to electron microscopic examination of the anti-AMP antibody-GS complexes, stabilization of the dodecameric GS structure by covalent cross-linking agents has been studied. Nearly complete stabilization is achieved by reaction of GS with high concentrations of carbodiimide at pH 6.0. Reactions with low concentration of carbodiimide or with either dimethylsuberimidate or glutaraldehyde yield incomplete cross-linkage. SDS-disc gel chromatography of these partially cross-linked preparations yields at least 10 different protein bands representing molecular species with molecular weights ranging from 50,000 to 600,000.

(iv) Glutamine Synthetase Regulation in Pseudomonas fluorescens. The concentration of glutamine synthetase in Pseudomonas fluorescens is 4 to 5 times greater than that found in E. coli grown under similar derepressing (nitrogen-limiting) conditions. Although activity of the enzyme is regulated by an adenylylation cascade, metabolic regulation of this cascade appears to be different from that previously demonstrated in E. coli, since an unconventional pattern of adenylylation of the enzyme is observed when the organism is grown on either glycerol, succinate, malate or fumarate. To facilitate further studies, the glutamine synthetase from P. fluorescens has been purified to homogeniety and many of its properties have been determined. This glutamine synthetase is significantly larger than the enzyme from E. coli (740,000 vs 600,000) and the pH-activity profiles of the adenylylated and unadenylylated forms in the presence of Mn^{2+} and Mg^{2+} are also different from the E. coli enzyme. From the pH profiles, a method of estimating the number of adenylylated subunits per enzyme molecule has been developed.

(v) The Glutamate Dehydrogenase Cascade of Candida utilis. Studies on the regulation of yeast NAD-dependent glutamate dehydrogenase (NAD-GDH) have continued. Both the active, dephospho- form (GDHa), and the less active phospho- form (GDHb) have been purified to homogeniety. They have identical amino acid compositions and a subunit size of 116,000 daltons, and normally exist either as a dimer or a tetramer. Because the affinity of GDHb for L-

glutamate (Km = 114 mM) is appreciably lower than that of GDHa (Km = 34 mM) it is unlikely that GDHb is catalytically active under physiological conditions.

Two cyclic nucleotide independent protein kinases, capable of phosphorylating GDHa, have been partially purified from extracts of C. utilis. However, the phosphorylation of GDHa by either of these kinases does not lead to inactivation of the enzyme. Both kinases preferentially phosphorylate acidic proteins such as casein and phosvitin, and are totally inactive with protamine histones, and bovine serum albumin.

(vi) Regulation of Microtubule Assembly. By taking advantage of the unique colchicine binding properties of tubulin, a new method for measuring the polymerization of tubulin has been developed. Compared to older techniques, the new method is more rapid and can be used to follow the kinetics of polymerization. By means of this new method, it has been found that the presence of fluoride cAMP and cGMP inhibit polymerization of tubulin in crude supernatants prepared from pig brain homogenates. The ATP supported polymerization is more sensitive to cyclic nucleotide inhibition than is GTP-dependent polymerization. Since cAMP has no effect on the polymerization of cycle purified tubulin, it appears either that purification of tubulin separates it from regulatory components, or that the fraction of tubulin isolated by the cycle procedure is insenstive to cyclic nucleotide control. Inhibition of polymerization by ATP and ADP, but not by AMP, can be overcome by sodium fluoride. In crude homogenates adenosine also inhibits polymerization. Results of studies with cAMP-dependent protein kinase inhibitor suggests that the cAMP-dependent inhibition of polymerization may involve protein phosphorylation. Initial experiments with ^{32}P demonstrate that the patterns of endogeneous protein phosphorylation in the crude supernatant fraction is markedly affected by fluoride, cAMP, and adenosine.

C. Metabolism of Branched Chain Amino Acids

The conversion of α-leucine to β-leucine is the first step in a newly discovered pathway of leucine metabolism. Leucine 2,3-aminomutase which catalyzes the interconversion of α-leucine and β-leucine has been found in higher plants, yeast, bacteria, liver, and leukocytes. The enzyme from all sources requires adenosylcobalamin for activity and is stimulated by FAD, coenzyme A, NAD, and pyridoxal phosphate. The maxim that plants and yeast do not contain corrinoids was negated by the use of a highly sensitive assay procedure which shows that both organisms contain significant amounts of cobalamin. Because cobalamin is required for the activity of leucine 2,3-aminomutase, it was proposed that the presence of high levels of β-leucine in the serum of patients with untreated pernicious anemia might be due to their inability to convert β-leucine to α-leucine. This presupposes that β-leucine is not derived solely from α-leucine. The possibility that β-leucine is derived from oxidation products of branched chain fatty acids was supported by the demonstration that α-leucine accumulated during the oxidation of isomy-

ristic acid by extracts of rat liver, and that this accumulation was prevented by the presence of intrinsic factor. However, contrary to expectation, β-leucine did not accumulate in the presence of intrinsic factor.

D. Mechanisms of Enzyme Action

(a) Alkaline Phosphatase

A detailed analysis of steady-state kinetic studies on the mechanism of action of E. coli alkaline phosphatase has been completed. The flip-flop model used to explain half of the sites reactivity is contrary to alternative substrate and product patterns. The effect of Tris on the k_{cat} of this enzyme can be accounted for by the rate of dissociation of the Tris-phosphate product.

(b) Asparaginase

Direct evidence for the occurrence of a covalent aspartyl-enzyme intermediate has finally been obtained. Incubation of [^{14}C]-asparagine with asparaginase at pH 5.5, 4° C, led to the production of a ^{14}C-labeled enzyme derivative which was not dissociated by precipitation with trichloroacetic acid, or Sephadex gel filtration. Treatment with pepsin led to the formation of a ^{14}C-labeled peptide fragment whose composition is under investigation.

(c) Asparagine Synthetase

The kinetic mechanism of asparagine synthetase is shown to be consistent with a hybrid Uni Bi-Ter Ping-Pong-Theorell-Chance mechanism with abortive complexes. The "two-site" Ping-Pong mechanism has been excluded.

(d) Theoretical Studies

A set of formulas for the rapid computation of the number of King-Altman patterns for the derivation of steady-state rate equations was previously reported. Though not mathematically proved, the validity of these formulas was verified by comparison of the results with those by conventional methods. Rigorous mathematical proof has now been obtained by consideration of the theory of combinations. In addition, a method for calculating such patterns in the presence of irreversible steps has been established.

(e) Glutamine Synthetase

A kinetic analysis of the irreversible inactivation of glutamine synthetase which occurs in the presence of ATP and L-methionine-S-sulfoximine indicates that an inactivated subunit in the dodecamer retards the reactivity of its neighboring subunits toward inactivation. From kinetic and direct binding studies, it has been established that the tight binding of L-alanine to the Mn^{2+} activated unadenylylated glutamine synthetase is due primarily to the strong synergism between ADP and L-alanine.

Annual Report of the
Section on Intermediary Metabolism
and Bioenergetics
Laboratory of Biochemistry
National Heart, Lung, and Blood Institute
October 1, 1978 to September 30, 1979

The research activities of the investigators in the Section on Intermediary Metabolism and Bioenergetics have been concerned with (1) the anaerobic metabolism of glycine and proline with particular reference to characterization of the protein components of the selenium-dependent glycine reductase system and the electron transport proteins that link proline reductase to NADH, (2) characterization of amino acid transfer nucleic acids (tRNAs) that contain selenium in the nucleoside portions of the molecules, (3) characterization of the Methanococcus vannielii proteins that constitute a formate-$NADP^+$ oxidoreductase system; the selenium, molybdenum, iron sulfur-dependent formate dehydrogenase and the 5-deazaflavin-dependent $NADP^+$ reductase, (4) isolation and identification of new selenoenzymes from Clostridium barkeri and Clostridium kluyveri, and (5) phosphate esterification and transfer reactions catalyzed by enzymes from Clostridium sticklandii.

Clostridial Glycine and Proline Reductases:

The anaerobic reductions of glycine and proline by their respective enzyme complexes serve as terminal electron sink processes for a number of anaerobic bacteria. Detailed study of these enzyme systems has uncovered several new and unusual biochemical phenomena. It was shown earlier that proline reductase contains 10 covalently attached pyruvate groups (one per each of the 10 subunits) each located in an N-terminal peptide joined to the subunit by an alkali-labile bond. Release of pyruvoyl peptides of identical composition by lithium borohydride reduction, by treatment with hydroxylamine or by mild alkali suggests a common point of cleavage at an ester linkage. Each of these peptides contains a pyruvate moiety at the blocked N-terminal position and the same carboxy terminal amino acid. If the existence of an ester linkage of this type can be more rigorously proven then the question of the mechanism by which this occurs in the formation of proline reductase will be very interesting to pursue. Recent experiments indicate that the protein B component of glycine reductase also contains covalently attached pyruvate. In view of the results with proline reductase it will be of interest to examine this protein for the presence of a similar pyruvoyl peptide, particularly since a number of clostridia contain both proline and glycine reductases.

The reduction of glycine to acetate and ammonia by glycine reductase results in the concomitant synthesis of ATP. The 12,000 dalton selenoprotein A component of glycine reductase is considered a likely component, from the chemical point of view, to serve as intermediate carrier for the orthophosphate that is esterified during the reaction and subsequently transferred to ADP to form ATP. For example if a cysteine or selenocysteine residue of the protein became phosphorylated to form an S-P or Se-P ester, subsequent transfer to ADP to form ATP could occur readily. Studies with model thiophosphate esters and development of methods to detect the postulated phospho-selenoprotein intermediate have been initiated and appear promising.

The preliminary finding that an unusual quinone-dependent phosphatase purified from C. sticklandii exhibits some activity with phosphocasein as substrate suggests that a bacterial phosphoprotein may be its natural substrate. Previously the only known substrate of this interesting enzyme was a synthetic ester, p-nitrophenylphosphate. A consideration of the properties of this enzyme suggest that it is more likely to act in the cell as a transferase rather than a phosphatase. The possibility of its interaction with the glycine reductase phosphorylated intermediate as well as with other phosphoproteins of C. sticklandii will be examined. The latter approach is now feasible because crude extracts can be freed of the phosphatase under very mild conditions by passage over an affinity column containing an attached quinone analog. Subsequent enzymic phosphorylation of these extracts with radioactive phosphate should provide a source of the natural substrate for the enzyme. Such studies may indicate a role of the phosphatase in a membrane transport process or in the regulation of a reaction catalyzed by the microorganism.

Earlier studies on the mechanisms of the glycine and proline reductase reactions were carried out using dithiols as electron donors but in vivo the reducing equivalents generated by oxidation of various substrates are transferred to NAD^+ and the resulting NADH is used as electron donor for glycine and proline reduction. Neither of the two reductases utilizes NADH directly; rather other proteins, also membrane associated, serve as intermediate electron carriers between NADH and the reductases. A 250,000 dalton iron protein that is required to link proline reductase to NADH has been isolated in pure form and shown to be a glycoprotein. The identities of the attached glycosyl groups, after hydrolysis and derivatization, are determined by gas chromatography. Although glycoproteins were once considered to be a rarity in bacteria, both the glycine reductase selenoprotein A and the iron protein of the proline reductase complex are glycoproteins. In view of the fact that these proteins interact with membrane associated enzymes their glycosyl group content is no longer a surprising finding.

Selenium Biochemistry:

Studies which had as their aim the development of a simple in vitro system in which to investigate the mode of biosynthesis of the glycine reductase selenoprotein A, instead led us to the discovery that certain anaerobic bacteria incorporate selenium into the nucleotide portion of some of their amino acid transfer nucleic acids (tRNAs). The process is highly specific for selenium and in C. sticklandii three readily separable seleno-tRNAs are formed. One of these is an L-prolyl-tRNA. The other two species are in the process of being identified. Compositional studies of these tRNAs labeled with ^{75}Se indicate the presence of a selenium modified nucleoside. Since ^{75}Se-labeled tRNA is produced by permeabilized cells and also in the presence of antibiotics that block protein synthesis, detailed studies on the mechanism of selenium incorporation into these molecules are feasible.

Selenium occurs in the form of selenocystein in three of the presently known selenoenzymes but the biochemical mechanism of incorporation of this unusual amino acid within a polypeptide chain is still unknown. In general the unusual amino acids found in proteins are formed by posttranslational modifications of the common amino acids that were inserted as the polypeptide

chain was formed. Also since no genetic code word is known for selenocysteine it is reasonable to expect that this selenoamino acid in a protein is produced by relatively simple chemical transformations of a preexisting serine or cysteine residue. Such a transformation, of course, would be enzymically specified in a very exact manner. Actually, in a number of experiments with C. sticklandii, ^{75}Se-selenocysteine was found to be a much more effective source of selenium for ^{75}Se-protein A synthesis than either ^{75}Se-selenite or ^{75}Se-selenide. Since this suggested the possibility that the intact amino acid might be incorporated as such, both ^{14}C and ^{3}H labeled selenocysteines were prepared enzymically and tested as sources of the carbon skeleton of the selenoamino acid residue in protein A. Only traces of ^{14}C or ^{3}H were found in the selenoprotein A but the results of these experiments were inconclusive because the administered labeled substrates were actively metabolized by the organism and selenium was rapidly eliminated into the medium. Although selenoprotein A synthesis by C. sticklandii is inhibited by antibiotics that block either protein synthesis or DNA-dependent RNA synthesis it is premature to conclude on the basis of these observations that the selenoamino acid is incorporated as such into the growing polypeptide chain. A survey of other anaerobic bacteria that have been recently discovered to produce selenoenzymes showed that some of these microorganisms do not rapidly decompose added selenocysteine and therefore, similar labeling experiments will be undertaken with these systems. One of these organisms, Clostridium barkeri, produces a selenium-containing nicotinic acid hydroxylase and another, Clostridium kluyveri, a high molecular weight seleno-iron sulfur protein. Alternatively, similar studies can be carried out with Methanoccus vannielii which produces a high molecular weight selenium-dependent formate dehydrogenase that was shown to contain selenocysteine.

Formate-$NADP^+$ Oxidoreductase of Methanococcus vannielii:

A conspicuous blue-green fluorescent cofactor present in methane bacteria was recently identified by investigators at the Univ. of Illinois, the Univ. of Nijmegen, and MIT as a 5-deazaflavin. An apparently identical substance isolated from M. vannielii is reduced by the formate dehydrogenase of this organism and serves as cofactor for the reductase that transfers reducing equivalents to $NADP^+$ to form NADPH. The reductase was purified to homogeneity, a number of its properties were determined and an independent fluorometric assay was developed for measurement of its activity. The reductase is somewhat active with a few closely related synthetic 5-deazaflavin analogs but not with common flavins such as FMN or FAD. Of particular interest to investigate is (a) the mode of biosynthesis of the 5-deazaflavin, e.g. how is a CH_2 group introduced in place of the N that occurs in the corresponding 5-position of the flavin ring system? and (b) do reductases directly responsible for methane biogenesis utilize this unusual 5-deazaflavin as cofactor or must reducing equivalents all be channeled through NADPH? Previous studies showed that there is a direct correlation between rate of growth of M. vannielii and availability of sufficient selenium in the medium to produce the selenium containing formate dehydrogenase complex. In selenium deficient media a smaller molecular weight formate dehydrogenase that contains molybdenum and iron sulfide but no selenium predominates. Whether these two types of formate dehydrogenases can couple equally well to the 5-deazaflavin dependent $NADP^+$ reductase to generate

NADPH for biosynthetic reactions is not known. Preferential utilization of the selenoenzyme either for NADPH formation or as electron donor for methane biosynthesis --- the energy yielding process for the organism --- could account for the increased rate of growth in the presence of selenium.

Annual Report of the
Section on Protein Chemistry
Laboratory of Biochemistry
National Heart, Lung and Blood Institute
Oct. 1, 1978 through Sept. 30, 1979

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

Glutamine synthetase (GS), a strictly regulated enzyme in E. coli, is a dodecamer with twelve catalytic sites. Interactions of divalent cations, substrates, and inhibitors with glutamine synthetase from E. coli have been studied by microcalorimetry, equilibrium dialysis, pH, ultracentrifugal, spectral, and kinetic techniques. Calorimetrically measured heats have provided information on the separateness of ligand binding sites and on proton uptake or release in binding reactions. Thermal saturation curves for the binding of low-affinity ligands to the enzyme have given thermodynamic binding parameters. A reversible thermal transition (observed by UV difference spectral measurements) is being used to study Me^{2+} and ligand stabilization of glutamine synthetase structure. The thermal transition occurs at high temperature over a narrow temperature range and appears to involve the exposure of one catalytically essential tryptophanyl residue per subunit; subunit dissociation does not occur during the annealing process. Spectrophotometric and sedimentation velocity measurements show that unadenylylated and adenylylated glutamine synthetase from E. coli undergo different conformational changes upon binding L-methionine-SR-sulfoximine, a proposed transition state analog. Binding L-methionine-SR-sulfoximine to unadenylylated enzyme complexes (Mn·GS, Mg·GS, and ADP·Mn·GS) produces red-shifted UV difference spectra consistent with the burial of approximately one tyrosyl residue per unadenylylated subunit. This may involve primarily the tyrosyl residue at the site of adenylylation because the binding of L-methionine-SR-sulfoximine to the fully adenylylated manganese-enzyme produces a red-shifted spectral perturbation of covalently bound 5'-adenylate groups without concomitant tyrosyl perturbations. Furthermore, the binding of L-methionine-S-sulfoximine phosphate + ADP at catalytic sites of glutamine synthetase blocks adenylyltransferase-catalyzed adenylylation of glutamine synthetase. Spectrophotometric results indicate also that both the S- and R- isomers of L-methionine-SR-sulfoximine bind reversibly with about the same affinity to unadenylylated glutamine synthetase and compete for a single subunit site. Negative cooperativity is observed in binding this analog to either the unadenylylated or adenylylated enzyme. Sedimentation velocity measurements show that unadenylylated and fully adenylylated manganese-enzymes have essentially the same conformation in the absence of L-methionine-SR-sulfoximine. However, the binding of this ligand to these glutamine synthetase forms produces small but different alterations in hydrodynamic particular shape. The binding of Mn^{2+}

to unadenylylated and fully adenylylated glutamine synthetase is the same. With either enzyme form, the binding of L-methionine-SR-sulfoximine enhances ∿30 fold the affinity of high-affinity Me^{2+} sites for Mn^{2+} --- possibly by a direct interaction. The substrate L-glutamate (but not L-glutamine) also influences the environment of Mn^{2+} bound to high-affinity n_1 metal ion sites and, furthermore, produces a small tyrosyl residue perturbation. These effects of L-glutamate binding on the enzyme conformation are being studied more quantitatively. Kinetic studies on Mn^{2+} interactions with glutamine synthetase show that the enzyme is converted from a low-affinity to a high-affinity metal ion form during Mn^{2+} binding, that the proton release occurring during the conformational change ($t_{1/2} \simeq 4$ min at 15°C) is due to a pK_a perturbation rather than to a direct replacement by Mn^{2+}, and that the release of Mn^{2+} from high-affinity sites has a half-time of ∿1 sec at 15°C. In addition, a novel reaction catalyzed by the unadenylylated manganese-enzyme has been discovered; this reaction is an AMP-supported synthesis of pyrophosphate and L-glutamate from orthophosphate and L-glutamine.

Calorimetric and ultracentrifugal studies with aspartate transcarbamoylase (ATCase) from E. coli have been performed in collaboration with H. K. Schachman (U.C., Berkeley, CA). The enthalpy change for the substrate-promoted transition ($\Delta H_{T\rightarrow R}$) of ATCase has been estimated by two different calorimetric approaches. The high-affinity bisubstrate analog N-(phosphonacetyl)-L-aspartate(PALA) and 40 mM phosphate buffers at pH 7.0 were used for these studies. The estimates of $\Delta H'_{T\rightarrow R}$ were made from measurements of: $\Delta(\Delta H')$ of PALA binding to ATCase (the difference between $\Delta H'$ values for binding PALA during and after completion of the transition) and the heats of assembly of ATCase (C_2R_3) from catalytic chain trimers (C) and regulatory chain dimers (R) in the absence and presence of PALA (together with $\Delta H'$ for PALA binding to C). Ultracentrifugation was used to measure the extent of the PALA-promoted conformational transition in ATCase and the ATCase formed in assembly reactions from isolated catalytic and regulatory subunits. Our results indicate that $\Delta H_{T\rightarrow R}$ is negative and small (∿-2 kcal/mol) with an attendant small proton uptake of ∿1 equiv of H^+ per mol of ATCase. The entropy change ($\Delta S_{T\rightarrow R}$) is negative and small (∿ - 20 cal/deg·mol) since $\Delta G_{T\rightarrow R} = 3.3$ kcal/mol ATCase. The assembly of ATCase (C_2R_3) from C and R is less favored in the presence than in the absence of PALA. The heats of assembly (in kcal per mol of catalytic:regulatory chain contact) of the unliganded T-state is ∿ - 13 and of the PALA-liganded R-state is ∿ - 9. Our results also show that $\Delta H'$ for binding PALA to free catalytic chain trimer is about twice as negative as that for binding PALA to catalytic chains in the intact ATCase molecule, suggesting an important role of regulatory chains in determining the latter thermodynamic quantity.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00201-08 LB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Metabolism of the Branched-Chain Amino Acids

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

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

COOPERATING UNITS (if any)

Dr. Carl M. Stevens, Washington State University, Pullman, Washington
Dr. Rose Sheinin, University of Toronto, Toronto, Ontario, Canada

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A study of the metabolism of the branched-chain amino acids has revealed a pathway of catabolism of leucine in which the first step is catalyzed by leucine 2,3-aminomutase. The enzyme, found in bacteria, plants, yeast, and man, is dependent upon adenosylcobalamin and is stimulated by FAD, coenzyme A, NAD, and pyridoxal phosphate. The relation between enzyme activity and various disease states will be examined.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The catabolism of the branched-chain amino acids, leucine isoleucine, and valine remains incompletely understood. Although the study of certain inborn errors of metabolism, e.g., maple syrup urine disease and isovaleric acidemia, has given some insight into the metabolism of these amino acids, additional information has been derived from studies of bacterial fermentation. 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 reported previously, 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:

α-leucine ⟶ β-leucine ⟶ β-keotisocaproate ⟶ acetate + 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 and leukocytes.

As reported last year, pure D-β-leucine and pure L-β-leucine were tested as substrates, and the D isomer was found to yield the greater amount of α-leucine as product. In order to see whether the optical isomerization of the product α-leucine had any relation to the optical nature of the substrate, extensive experiments were conducted. Establishment of the optical properties of the product proved to be extremely difficult; technical problems, including limited amounts of substrate, hampered the research. Results indicate, however, that the product α-leucine may be a racemic mixture, regardless of the optical form of the substrate β-leucine.

The corrinoid cofactor which has been isolated from potato tubers (reported last year) was examined in an isotope dilution assay using intrinsic factor and radioactive cyanocobalamin. Two separate preparations made from potatoes grown in different locales both exhibit competition with authentic cobalamin. This strongly supports the contention that these cofactors are true cobalamin.

Yeasts, like plants, have been thought to be devoid of corrins. The finding of cobalamin in potatoes led to the examination of the yeast, Candida utilis. It was found to contain quantities of cobalamin on the order

of 5 pmol per gram of moist cell paste, principally as the methyl and adenosyl derivatives. In addition, the yeast was shown to possess the methylcobalamin-dependent system for methionine biosynthesis and the cobalamin-dependent enzyme, leucine 2,3-aminomutase. Thus, it can be concluded that yeasts, as a class, are not unique in their metabolism.

The finding that patients with untreated pernicious anemia, who are therefore deficient in cobalamin, have elevated levels of serum β-leucine led to the examination of the probable source of the β-amino acid. If the leucine 2,3-aminomutase is inactive, α-leucine cannot be the source. Therefore, consideration must be given to mechanism of production of the β-amino acid or its probable precursor, β-ketoisocaproic acid. This latter acid might arise from the beta oxidation of branched-chain fatty acids of the iso-series. In order to test this hypothesis, crude extracts of rat liver were incubated with isomyristic acid. A substrate-dependent production of α-leucine was obtained. This production was inhibited by the addition of the cobalamin-specific binder, intrinsic factor, and the inhibition could be overcome by the addition of exogenous cobalamin. These findings are consistent with the proposed source of the β-amino acid.

Certain strains of temperature-sensitive mouse tissue culture cells developed at the University of Toronto by Dr. Rose Sheinin and her colleagues have been proposed as animal models for megaloblastic anemia on a morphologic basis. In order to establish whether these cells exhibit biochemical differences that might be similar to those seen in cobalamin deficiency, cell-free extracts of these cells are being examined for leucine 2,3-aminomutase activity. It is not yet clear whether there is a difference in the metabolic activity that corresponds to the morphological difference. The cell-free extracts, however, exhibit atypical responses to exogenous cobalamin in that leucine 2,3-aminomutase activity is decreased when the cobalamin is added. The significance of this is yet to be determined.

Proposed Course of Action

The leucine mutase will be purified and characterized. To this end, the conditions which yield maximum activity in cell cultures will be established and sources which may yield stable enzyme will be examined. The nature of the B_{12} involvement will be established and the nature of the other cofactor involvement will be examined. The distribution of the pathway in normal and diseased mammalian and human tissues will be studied as will its relation to plant development. There will be examination of the relationship of the pathway to lipid metabolism. The other enzymes in the pathway will be examined and their cofactor requirements established.

Relevance to Biomedical Research

This study impinges on at least three areas of medical concern: (1) the mode of action of vitamin B_{12} in its metabolic roles; (2) the means by which organisms catabolize food materials; and (3) the probable nutritional

value of plant material with regard to 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, isovaleric acidemia, and disorders of the catabolism of short-chain acids. The mode of action of B_{12} is imperfectly understood, but its importance in hematopoiesis and in the maintenance of proper neurological function is exemplified in the disease of its metabolic deficiency, pernicious anemia.

Publications

None

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October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Kinetics, Regulation and Mechanisms of Biochemical Reactions

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

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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)

(1) Theoretical analysis of the cyclic cascade model reveals that in addition to the properties described in the previous report, the modification and demodification of the last interconvertible enzyme in the cascade proceeds with a lag, and this lag time is dependent on the number of cycles involved in the cascade. The magnitude of the rate amplification is dependent on the number of cycles, kinetic constants, and the concentration of both effectors and enzymes involved. The adenylylation-deadenylylation of glutamine synthetase system and the phosphorylation dephosphorylation of protein by c-AMP-dependent protein kinase and phosphatase is being studied for the verification of the cyclic cascade model. (2) The irreversible inhibition of glutamine synthetase by L-methionine-S-sulfoximine reveals the homologous subunit interaction in the enzyme. The catalytic cycle for alkaline phosphatase was elucidated; and the catalytic cycle for the adenylylated glutamine synthetase using the fluorescence signal of 2-aza-adenylylated enzyme is being investigated. (3) Kinetic studies of L-Ala binding reveal that the tight binding of L-Ala to the Mn(II) activated unadenylylated glutamine synthetase is primarily due to the strong synergism between ADP and L-Ala. Other studies included CRO protein-DNA interaction and actomyosin ATPase.

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Project Description

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

Major Findings

I. Covalent Modification and Cellular Regulation of Enzymic Activity

A. Theoretical Analysis of Cyclic Cascades Systems. Analysis of cyclic cascades which are derived from the dynamic coupling of two opposing unidirectional cascades was continued. The effort was concentrated on the exploration of the time-dependent properties. In the analysis, the differential equations were numerically integrated. The results showed that: (1) covalent modification of the last interconvertible enzyme in a cyclic cascade, which contains more than one cycle, proceeds with an initial lag. The lag time increases as the number of cycles increases in the cascade. Following the initial lag, a burst in the formation of the modified interconvertible enzyme takes place. The steepness of this burst increases as the number of the cycles in the system increases. This rapid acceleration in rate is derived from the fact that the rate of covalent modification of the last interconvertible enzyme in the cascade is a multiplicative function of the rate constants of all the reactions that lead to the formation of the modified enzyme. Thus, the cyclic cascade is a rate amplifier. (2) The magnitude of the rate amplification as a function of the number of cycles involved in the cascade can vary enormously, depending on the kinetic constants and concentration of both effectors and enzymes involved. For example, the rate amplification is much more pronounced when the concentration of the interconvertible enzyme increases with each successive cycle in the cascade, such that a pyramidal relationship in concentration for the interconvertible enzymes is maintained. In fact, this pyramidal relationship is often found in vivo. (3) The rate of modification of the last interconvertible enzyme is dependent on the concentration of the primary effector, an effector which triggered the cascade, and this rate is more dependent on the number of cycles in the cascade at lower primary effector concentration. (4) The rate of regenerating the unmodified interconvertible enzyme caused by the removing of the primary effector is also dependent on the number of cycles in the cascade. The regeneration time course proceeded with a lag time and this lag time is longer as the number of cycles in the system increases.

The results of this kinetic analysis, coupled with the steady-state analysis reported earlier, reveal that the cyclic cascade system is a superior regulatory mechanism. Therefore, it is not surprising that it is widely used to regulate biological activity.

B. Experimental Verification of the Predicted Cyclic Cascade Properties
Studies on the adenylylation-deadenylylation of glutamine synthetase has demonstrated the validity of the predicted properties for the monocyclic cascade system. A more detailed study of this covalent modification cascade is currently in progress. The initial stage of this program is the large scale purification of the proteins involved in the cascade, such as adenylyltransferase, modified and unmodified regulatory P_{II} proteins, P_{IIA} and P_{IID}, uridylyltransferase, and uridylyl-removing enzyme. In addition, the cyclic cascade system involving phosphorylation-dephosphorylation of proteins is also being investigated. In this case, bovine heart c-AMP-dependent protein kinase and phosphatase are used. The substrate for the kinase is histone HI from rabbit thymus. Purification of these proteins is currently in progress.

II. Physical and Mechanistic Studies of Enzymes

A. Subunit Interaction in Unadenylylated Glutamine Synthetase, Evidence Derived from Methionine Sulfoximine Inhibition Studies. Although glutamine synthetase from Escherichia coli is composed of 12 identical subunits, there is no evidence that homologous subunit interaction occurs in fully unadenylylated or fully adenylylated enzyme. Meister and coworkers (Biochemistry 8, 2681, 1969) have shown that L-methionine-S-sulfoximine, one of the four stereoisomers of methionine sulfoximine, preferentially inhibits glutamine synthetase irreversibly in the presence of ATP, due to the formation of the tightly bound products, ADP and methionine sulfoximine phosphate. Using highly purified unadenylylated glutamine synthetase and the two resolved enantiomers of L-methionine-S,R-sulfoximine, we have studied the kinetics of glutamine synthetase inactivation in the presence of excess methionine sulfoximine and ATP. The reaction of L-methionine-S-sulfoximine with glutamine synthetase in the presence of excess ATP can be described as

$$E\cdot ATP + MS \overset{K_1}{\rightleftharpoons} E\cdot ATP\cdot MS \overset{k_2}{\longrightarrow} E\cdot ADP\cdot MS\text{-}P$$

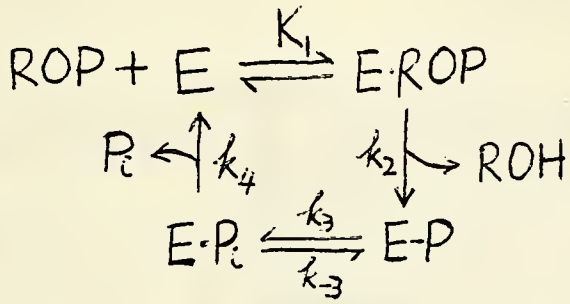
where MS is methionine sulfoximine and MS-P is methionine sulfoximine phosphate, K_1 and k_2 are the dissociation and rate constant, respectively. The binary complex, E·ATP, is in rapid equilibrium with the enzyme and ATP, and under the experimental conditions, E is essentially saturated with ATP. When the concentration of MS ued is in large excess relative to [E], the rate of E·ADP·MS-P formation is

where $k_{app} = \frac{k_2 [MS]_o}{K_1 + [MS]_o}$, $[E \cdot ATP]_o$, and $[MS]_o$ are initial concentrations of E·ATP and MS, respectively. Equation (1) predicts that the inactivation should follow a first order process under the experimental conditions. However, the observed kinetic patterns show that the inactivation slows down progressively from the expected first order rate. This indicates than an inactivated subunit in the dodecamer retards the reactivity of its neighboring subunits toward methionine sulfoximine and ATP. Since k_{app} consists of both K_1 and k_2, the observed negative cooperativity can be derived from the effect of either K_1 or k_2, or both K_1 and k_2. Strong negative cooperativity has been observed for the binding of methionine sulfoximine to both unadenylylated and adenylylated enzyme in the absence of nucleotide (J. Supramole. Struc., Supple. No. 3, page 101, Abst. No. 250, 1979). Therefore it is reasonable to assume that affinity (K_1) of GS for methionine sulfoximine also changes in the presence of ATP. However, this change in K_1 may not be sufficient to explain the observed decrease in k_{app} because K_1 has been estimated to be about 100 μM which is significantly lower than the concentration of L-methionine sulfoximine used (1 and 3 mM). Thus, the observed decrease in k_{app} is likely due to changes in k_2 values. In a separate experiment, the rate of ^{32}P incorporation into glutamine synthetase due to formation of tightly bound methionine sulfoximine [^{32}P]-phosphate was found to be slower than the loss of γ-glutamyl transferase activity of the enzyme. This suggests that formation of methionine sulfoximine phosphate and ADP on a specific subunit not only inactivates that subunit irreversibly, but also reduces the γ-glutamyltransferase activities of its neighboring subunits. This hypothesis is also supported by the observation that after incubation of glutamine synthetase with methionine sulfoximine and ATP, followed by exhaustive dialysis, only 0.75 equivalents of ADP is bound per subunit, even when only < 5% γ-glutamyltransferase activity remains.

Meister and coworkers (Biochemistry 8, 2681, 1969) reported that only the L-methionine-S-sulfoximine diastereomer inhibits the enzyme. Our results show that the L-methionine-R-sulfoximine binds to glutamine synthetase, but cannot react with ATP. Binding of the R-isomer will protect the enzyme temporarily from the irreversible inhibition of the S-isomer.

B. Kinetics and Mechanism of E. coli Alkaline Phosphatase. Alkaline phosphatase is an enzyme consisting of 2 identical subunits. At alkaline pH's, both the initial rate and phosphate binding data show that the enzyme exhibits a strong negative cooperativity. In order to provide a role for the second subunit, a flip-flop mechanism has been proposed (Eur. J. Biochem. 20, 124, 1971). However, steady-state kinetic data obtained with alternative substrates are in agreement with a simple negative cooperativity model, instead of the proposed flip-flop mechanism. To further explore the mechanism of this enzyme, several attempts have been made to elucidate the detailed mechanism at alkaline pH's. Due to the inability to monitor the rate of the rapid alcohol formation in the initial phase of the catalytic cycle, the mechanisms of alkaline phosphatase at alkaline pH has not been agreed upon

among the workers in the field. Utilyzing a custom built fluorescent stopped-flow machine which is equipped with a fast mixing cell, we monitored the initial burst of the alcohol formation. In this study, the substrate used is a nonfluorescent 4-methyl umbelliferyl phosphate which yields a highly fluorescent alcohol, 4-methyl umbelliferone, upon hydrolysis. The reaction proceeds via a rapid initial burst followed by a steady-state rate. The simplest catalytic scheme required to describe the catalytic cycle is shown in Scheme I.



Scheme I

where E, ROP, ROH, E-P, and E·Pi represent the dimeric enzyme, substrate, alcohol, covalently phosphorylated enzyme and noncovalent enzyme-phosphate complex, K_1 is the dissociation constant and the lower case k represents rate constant for the designated step. Based on Scheme I and the assumption that E and ROP are in rapid equilibrium, the rate expression for ROH formation is

$$[ROH] = k_2 f_{ES} [E]_o \left[\frac{k_3 k_4}{\alpha_1 \alpha_2} t + \frac{\alpha_1^2 - \alpha_1(k_3 + k_{-3} + k_4) + k_3 k_4}{\alpha_1^2(\alpha_1 - \alpha_2)} (1 - e^{-\alpha_1 t}) + \frac{\alpha_2^2 - \alpha_2(k_3 + k_{-3} + k_4) + k_3 k_4}{\alpha_2^2(\alpha_2 - \alpha_1)} (1 - e^{-\alpha_2 t}) \right] \quad (2)$$

where $f_{ES} = \frac{[ROP]}{K_1 + [ROP]}$ and $[E]_o$ is the total enzyme concentration, t is time, α_1 and α_2 are the two roots of the equation

$$\alpha^2 + (k_2 f_{ES} + k_3 + k_{-3} + k_4)\alpha + k_2 f_{ES}(k_3 + k_{-3} + k_4) + k_3 k_4 = 0$$

Equation (2) indicates that the time course for the initial burst of ROH formation is consisted of two exponential functions. However, if $k_3 >> k_4$ and $k_{-3} \simeq 0$, Equation (2) can be reduced to a single exponential function as shown

in Equation (3)

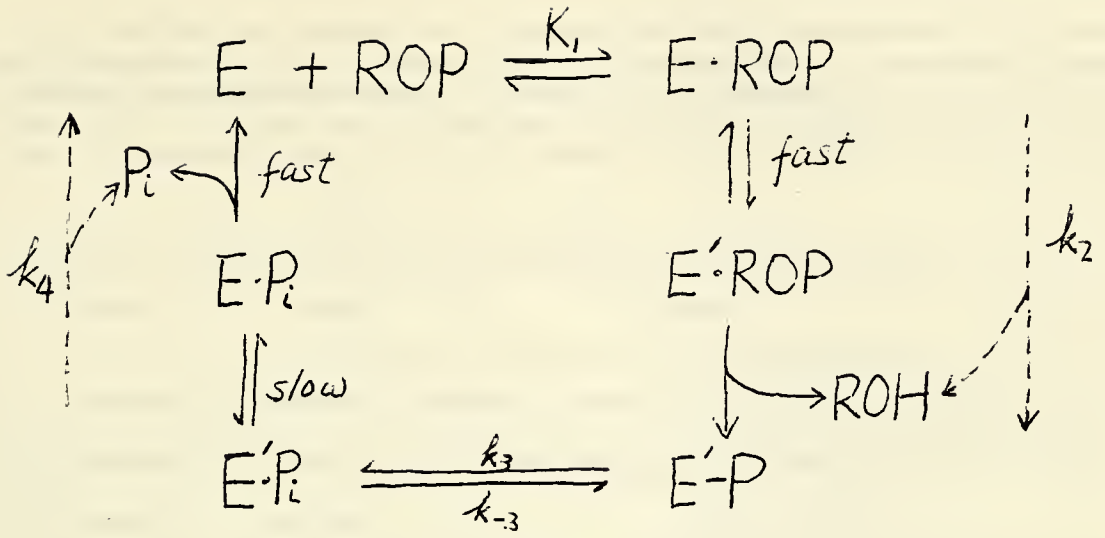
$$[ROH]=[E]_o\left[\frac{k_2 f_{ES} k_4}{k_2 f_{ES}+k_4}t+\left(\frac{k_2 f_{ES}}{k_2 f_{ES}+k_4}\right)^2\left(1-e^{-(k_2 f_{ES}+k_4)t}\right)\right] \quad (3)$$

The experimental data are in accord with Equation (3). In fact, the conditions which allow one to reduce Equation (2) to Equation (3) were fulfilled, since the values of k_3, k_{-3}, and k_4 were determined from separate experiments to be 78, ~ 0.2, and 7.6 sec^{-1}, respectively.

From the amplitude of the burst and Equation (3), it was determined that only one of the two potentially active sites is reactive at any one time when substrate concentration is less than 50 μM. This finding is in accord with the negative cooperativity observed for this enzyme, but is in disagreement with the "instant burst" data of Block and Schlesinger (J. Biol. Chem. 248, 5794, 1973) where they attained an amplitude equivalent to two reactive sites per dimer.

In addition to the value of k_3, which is independent of added substrate, and k_4, the kinetic data allowed us to calculate the values of K_1, k_2, and k_c, the overall catalytic rate constant, they are $\geq 1.5 \times 10^{-4}$ M, ≥ 2400 sec^{-1}, and 7.1 sec^{-1}, respectively. The results of the rate analysis for the catalytic cycle reveal that the rate-limiting step for the phosphate ester hydrolysis at pH 8.0 is the release rate (k_4) of the noncovalently bound inorganic phosphate from the enzyme. The value of k_4, 7.1-7.6 sec^{-1}, determined here, is in good agreement with the rate constant determined for the phosphate dissociation rate by ^{31}P NMR method in the absence of substrate, from which a rate constant ≤ 25 sec^{-1} was attained at 25° C in 0.1 M Tris, pH 8.0. When this constant is normalized to 10° C, a value of ≤ 11 sec^{-1} is obtained. Thus, facilitation of phosphate release by substrate binding to the second catalytic site, the essence of the flip-flop mechanism, is not observed.

It should be pointed out that Scheme I does not show the conformational change steps which are required for the explanation of negative cooperativity. A more realistic catalytic scheme, which takes into account both the kinetic data and conformational change steps, is given in Scheme II.



Scheme II

In this scheme, the conformational change step, which induces the dimer into a form (E') where substrate binding at the second site is retarded, is rapid. Therefore, this conformational change step is coupled with the ROH release step and its overall rate constant is expressed by k_2 of Scheme I (see dashed line of Scheme II). Similarly, the slow Pi release is likely to be involved with a slow conformational change step, since Pi released from most proteins proceeds with a rate constant > 10^4 sec^{-1}. Thus, the true rate-limiting step at alkaline pH is the conversion of E'·Pi to E·Pi, a step which is probably sensitive to ionic strength and to the nature of the bound phosphate, but independent of the nature of phosphate ester substrates.

C. Kinetic and Thermodynamic Study of L-Ala and D-Val Binding to the Unadenylylated Glutamine Synthetase in the Presence of Mn(II) and Ligands. Glutamine synthetase is regulated by both covalent modification and feedback inhibitors. In the presence of Mg(II), it has been shown that L-Ala, a feedback inhibitor, binds to an allosteric site which is different from L-Glu binding site on the unadenylylated enzyme. The dissociation constants determined for L-Ala when L-Ala complexes with E·ATP, E·ATP·NH_3, and E·ADP·AsO_4·NH_2OH in the presence of Mg(II) were 29 mM, 20 mM, 19 mM, and 3 mM, respectively. In the presence of Mn(II), steady-state kinetic data show that L-Ala behaves almost like a competitive inhibitor with respect to L-Gln in the Mn(II) supported γ-glutamyltransferase reaction. However, NMR data obtained in the presence of Mn(II) and ADP indicate that L-Gln and L-Ala bind at two separate sites on the unadenylylated enzyme. In addition, both the steady-state and NMR data indicate that the Mn(II) supported unadenylylated enzyme exhibits a very high affinity for L-Ala. To explore the origin of this unusually high affinity. Kinetic and thermodynamic parameters were measured for L-Ala binding to Mn(II) activated unadenylylated enzyme in the presence of various substrates (ADP, AsO_4, NH_2OH) for γ-glutamyltransferase reaction. Binding constants were measured by (i) sedimentation of the

protein in an air-driven centrifuge known as the airfuge and analyzing the unbound [^{14}C]-L-Ala in the supernatant; (ii) analysis of the amplitudes from the stopped-flow measurements derived from the protein fluoresence changes induced by L-Ala binding. The binding constants so determined for the reactions carried out at 15° C in 50 mM HEPES (pH 7.2) in the presence of 1 mM Mn(II) are:

Experiment	Reaction	K_{diss}	Method
1	E·ADP·AsO_4 + L-Ala	50 μM	Airfuge
2	E·ADP·AsO_4·NH_2OH + L-Ala	15 μM	Airfuge
3	E·ADP + L-Ala	500 μM	Stopped-flow
4	E·ADP·NH_2OH + L-Ala	200 μM	Stopped-flow
5	E·AsO_4 + L-Ala	20 mM	Stopped-flow amplitude
		28 mM	Stopped-flow kinetics
6	E + AsO_4	7.0 mM	Fluorometer
7	E·L-Ala (120 mM) + AsO_4	2.0 mM	Fluorometer
8	E·ADP + D-Val	0.65 mM	Stopped-flow amplitude
		0.47 mM	Stopped-flow kinetics

The data in experiments 1 and 3 indicate that the binding of AsO_4 would enhance L-Ala affinity by 10-fold; with this factor and the K_{diss} obtained in experiment 5, one can estimate the dissociation constant for Mn(II)·E·L-Ala to be ~ 250 mM which is in good agreement with the estimated K_{diss} of 300 mM reported previously (Biochemistry 14, 1980, 1975). This suggests that ADP binding enhances the L-Ala affinity by 500-fold (experiment 3) for the Mn(II) activiated unadenylylated enzyme. This clearly is the major contribution for the low K_i of L-Ala in the Mn(II) system. In addition to the synergistic effect between ADP, AsO_4 and L-Ala, NH_2OH also enhances the affinity for L-Ala (comparing experiments 1 and 2). It should be pointed out that only 3.5-fold instead of 10-fold difference in K_{diss} was observed between experiments 6 and 7. This is because the enzyme was not saturated with L-Ala.

Analysis of the stopped-flow data indicate that a minimum of two reaction steps is required to explain the kinetic of L-Ala binding. This mechanistic scheme is shown in Equation (4)

$$E + L\text{-}Ala \rightleftharpoons^{K_1} E \cdot L\text{-}Ala \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E^* \cdot L\text{-}Ala \qquad (4)$$

where K_1 is the dissociation constant, E and E^* indicate two different enzyme species which differ in their conformation. The value of $k_2 + k_{-2}$ has been computed to be 76 and 102 sec^{-1} for experiments 3 and 4, respectively. For experiment 5, K_1 is 28 mM and k_2 = 2.2 sec^{-1}, k_{-2} = 2.3 sec^{-1}.

The kinetic data for D-Val binding to E·ADP was also studied. The results reveal that D-Val binding also involves a minimum of two reaction steps as that shown in Equation (4). The value of K_1 was determined to be 1.4 mM and k_2 = 45 sec^{-1}, k_{-2} = 15 sec^{-1}. This yields an overall dissociation constant of 0.5 mM which is in reasonable agreement with the value of 0.8 mM determined by the NMR method.

D. Mechanistic Studies of 2-Aza-ϵ-Adenylylated Glutamine Synthetase. The catalytic cycle for the biosynthesis of glutamine catalyzed by the unadenylylated glutamine synthetase from E. coli has been elucidated utilyzing the intrinsic tryptophan fluorescence of the protein (Proc. Natl. Acad. Sci. U.S.A. 73, 476, 1976). However, this fluoresence change does not occur with the adenylylated enzyme upon addition of substrates. When the unadenylylated enzyme is adenylylated enzymatically with a fluorescence derivative of ATP, 2-aza-ϵ-ATP, the 2-aza-ϵ-adenylylated enzyme exhibits similar catalytic properties and inhibitor susceptibility to those of the naturally adenylylated enzyme. The excitation wavelengths for the 2-aza-ϵ-adenylylated enzyme are 300 and 368 mμ, and the emission maximum is at 470 mμ. The fluorescent properties of the 2-aza-ϵ-adenosine were used to study ligand-induced conformational changes. Various degrees of fluorescence changes were observed when the substrates for both biosynthetic and the transferase reactions were added individually or in a multiple manner to the modified enzyme in the presence of Mn(II). In the case of biosynthetic reaction, the kinetic study shows that the formation of an intermediate, which is formed in the presence of ATP and L-Glu, is the rate limiting step. This intermediate, as identified by its fluorescence spectrum, reacts rapidly with NH_3. The preliminary data for this type of study indicates that it is possible to elucidate the complete catalytic cycle for the adenylylated enzyme catalyzed biosynthetic reaction. This study is currently in progress.

E. Kinetic and Mechanism of Rabbit Skeletal Muscle Actomyosin ATPase Reaction. At low salt concentration (1.5 mM $MgCl_2$), and low temperature, 5° C, myosin subfragment-I (S-1) dissociates from the acto-S-1 complex and reassociates each time an ATP molecule is hydrolyzed by Acto-S-1. The rate-

limiting step in the catalytic cycle involves the conformational change of S-1 following its dissociation from actin. However, at higher temperature, e.g., 15° C, the stopped-flow data indicate that ATP, actin, and S-1 are in rapid equilibrium with S-1·ATP and Acto-S-1·ATP complexes. In addition, S-1·ATP and S-1·ADP·Pi exhibit the same affinity for actin. Thus, at high actin concentration both S-1·ATP and S-1·ADP·Pi are present as their respective actin complexes. If the dissociation of actin from Acto-S-1·ATP is required for ATP hydrolysis, and the rate-limiting step in the actomyosin ATPase cycle is the release of products from the actomyosin complex as proposed by Lynn and Taylor (Biochemistry 10, 4617, 1971), then at high actin concentration one expects an inhibition on ATPase activity. However, experimentally, inhibition of ATPase by high actin concentration was not observed either in the burst phase or steady-state phase of the reaction. The results suggest the the dissociation of S-1 from actin is not required for the hydrolysis of ATP. In other words, the rate-limiting step proceeds with the same rate whether S-1 is bound to or dissociated from actin.

F. Lambda CRO Protein-DNA Interaction. CRO protein specified by bacteriophage λ is a repressor protein of the gene expressed early in phage development and is required to carry out regulatory functions during the late stage of lytic growth. It is a dimeric protein with subunit molecular weight of 7350 daltons. Each subunit consists of three tryosyl residues. This fluorescence property was used to study the interaction between CRO protein and calf thymus DNA. The binding of CRO protein to DNA results in a 70% quenching for the tyrosine fluorescence. The binding constant, determined by fluorescence titration, was found to be dependent on monovalent and divalent cation concentrations. Kinetic measurement reveals that the association rate constant is greater than 10^8 M^{-1} sec^{-1}. This very high rate constant is likely due to the high number of base pairs in the DNA molecule. In other words, the interaction is probably due to nonspecific interaction between the CRO protein and the base pairs on the DNA. This point will be resolved by measuring the binding rate as a function of base pairs.

Significance to Biomedical Research

The overall object 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.

The cyclic cascade analysis reveals the importance of the covalent modification of protein in metabolic regulation. Based on the results of the analysis, it is likely that a cyclic cascade system is involved in the regulation of many key enzymes in metabolism, and it may also be involved in biological processes in which signal amplification or rate amplification is important, such as neural transmission or hormonal induced responses. Through the study on the covalent modification cascade, we hope to learn more about the basic principles involved in the regulation of glutamine synthetase by covalent modification, and some regulatory properties involving the phosphorylation of enzyme by c-AMP-dependent protein kinase and dephosphorylation by

phosphoprotein phosphatase.

The physical and mechanistic studies of enzymic action will enhance our knowledge on how enzymes function. This knowledge is instrumental in controlling the function of a specific enzyme. The elucidation of the catalytic cycle for alkaline phosphatase may provide a correction for the concept in explaining negative cooperativity.

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 c-AMP-dependent protein kinase and phosphoprotein phosphatase will be investigated.

(3) To further explore the physical, chemical, and immunological properties of unadenylylated and adenylylated glutamine synthetase. In particular, we shall 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.

Publications

Articles published in periodicals

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00203-06 LB

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Cellular Regulation of Enzyme Levels

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

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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:
1.4	1.1	0.3

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The purpose of this investigation is to study the regulation of intracellular protein degradation in Escherichia coli. In an effort to obtain cell-free perparations that proteolyze specific enzymes, extracts of E. coli were made by gentle disruption of cells with lysozyme-EDTA. Such extracts catalyze the degradation of added purified glutamine synthetase, as well as other purified proteins, and represent a proteolytic artifact due to lysozyme. This lysozyme-induced proteolysis has been studied using E. coli glutamine synthetase in order to characterize the essential features of protein-protein interaction required for hydrolysis. In addition, studies on intracellular degradation in E. coli have continued. Partial degradation of added purified glutamine synthetase has been demonstrated in cell-free extracts derived from catalase-deficient mutants, and these mutants represent a potential source for isolation and characterization of an intracellular degradation system in E. coli. Techniques used in these studies have included polyacrylamide pore gradient electrophoresis, isotopic labeling, chromatographic techniques, autoradiography, and enzymatic assay of functional proteins.

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Project Description

The control of intracellular enzyme level is important in metabolic regulation. Changes in these enzyme levels reflect either changes in the rate of protein synthesis at the transcriptional or translational level, or changes in the rate of degradation. The metabolic control of the various aspects of protein synthesis has been well characterized, but little is known about intracellular degradation or its regulation. The object of this investigation is to elucidate some of the basic mechanisms underlying the process of protein degradation. This study has been divided into two parts, namely, lysozyme-induced proteolysis of glutamine synthetase, and the isolation and characterization of an intracellular degradation system in E. coli.

Major Findings

I. We have previously shown that lysozyme-prepared E. coli cell-free extracts catalyze the degradation of added purified glutamine synthetase (GS). The proteolytic activity is due to the lysozyme used in the extract preparation. Incubation of ^{14}C-AMP-GS with lysozyme alone was accompanied by loss of label from 50,000 MW GS subunit on SDS polyacrylamide pore gradient gels. Trace hydrolytic activity was observed when lysozyme was incubated with casein, denatured hemoglobulin, p-nitrophenyl acetate, but not with a variety of synthetic protease substrates. Since this proteolytic activity could not be separated from the lysozyme lytic activity by physical techniques, including gel filtration, ion exchange chromatography and electrophoresis under a variety of conditions, we have concluded that this activity is an intrinsic property of lysozyme. The proteolytic activity is apparently associated with the specific esterase site on lysozyme-involving histidine-15 described by Piszkiewicz and Bruce (Biochemistry 7:3037, 1968). Selective carboxymethylation of histidine-15 by their method destroys both the esterase and the proteolytic activity, but the modified lysozyme retains 40% of lytic activity.

In early experiments, it was always possible to demonstrate loss of label from ^{14}C-AMP-GS subunits on SDS gels, or from TCA precipitable material when lysozyme was incubated with labeled adenylylated GS. However, it was difficult to recover the label from smaller protein species or TCA-soluble material, until it was found that this material was adsorbed by glass. Recoveries of 90% or better could be achieved by using plastic labware.

Additional studies were designed to study the specific interaction of lysozyme and GS. Since incubation of ^{14}C-AMP-GS with lysozyme alone was accompanied by loss of ^{14}C from the GS subunit on SDS gels, it was necessary to rule out the possibility that lysozyme possessed phosphodiesterase activity and functioned to cleave the AMP group from the adenylylated enzyme. PEI cellulose and cellulose thin layer chromatography of typical reaction mixtures with cold free AMP revealed label in multiple species, but no label migrated with free AMP. Comparing time course of incubation by SDS electrophoresis and TCA precipitation, it was found that label was lost from the GS subunit long before label appeared in the TCA soluble fraction. Although the labeled end product of the reaction was not identified, these experiments

suggest that a phosphodiesterase activity, if present, was not the first or primary step in the reaction sequence.

All of the early experiments were carried out at low lysozyme concentration, and only trace proteolytic activity was observed. Later it was found that the lysozyme/GS molar ratio was important, the optimum being 24/1. At this very high concentration of lysozyme, multiple protein staining species could be observed on SDS gels. Similar results were obtained when lysozyme was incubated with other proteins such as E. coli β-galactosidase avidin and bovine serum albumin. Incubation of either GS or β-galactosidase with lysozyme led to partial loss of activity. The reaction reaches a plateau and does not go to completion even with prolonged incubation. Further loss of activity is observed when more lysozyme is added to the reaction mixture, suggesting that the proteolytic activity of lysozyme is subject to product inhibition. When these reaction mixtures are subjected to SDS electrophoresis, complete loss of GS subunit is observed. Treatment of lysozyme with SDS, EDTA, heat, and urea does not rapidly inactivate the lysozyme esterase or proteolytic activity. However, pretreatment of the substrate GS with these agents followed by lysozyme treatment leads to rapid proteolysis indicating that the primary effects of these agents may be a combination of substrate denaturation which exposes susceptible cleavage sites and/or dissociation of inhibitory product (peptide) from an inhibited lysozyme complex. Also, high concentrations of ATP stimulate lysozyme-induced inactivation of GS and β-galactosidase, but the nature of this stimulation has not yet been determined.

II. It has been exceedingly difficult to demonstrate proteolysis or inactivation of exogenous GS by E. coli cell-free extracts derived from cells grown under a variety of nutritional conditions. Although concomitant proteolysis has not yet been demonstrated, a potent system capable of inactivating GS has been obtained from extracts of Klebsiella aerogenes. This inactivation is markedly inhibited by the addition of beef heart catalase. Subsequent examination of endogenous catalase levels revealed that while Klebseilla extracts were nearly devoid of catalase activity, E. coli extracts possessed high levels of catalase. Reduced catalase levels were observed in extracts derived from anaerobically grown E. coli, but since it was difficult to obtain reproducibly, low catalase levels catalase-deficient mutants were obtained.

In addition to the catalase-deficient mutant obtained in this laboratory which requires δ-amino levulinic acid for normal colony formation, another mutant (popA), which lacks ferrochelatase, activity was obtained from E. coli genetic stock maintained by Dr. Barbara Bachmann. Each of the catalase-deficient mutants possesses a different lesion in porphyrin biosynthesis and is therefore deficient in cytochromes as well as other porphyrin-containing proteins. Both of these mutants grow poorly under aerobic conditions, but will grow anaerobically in a medium containing yeast extract supplemented with glucose. When extracts of these cells were incubated with exogenous GS in the presence of ATP, NADPH and iron, loss of GS subunit protein was observed on SDS gels as compared with unincubated controls. In addition, there was an apparent enhancement of two bands in the region of 35,000 and 17,000

molecular weight. Since the protein background was high on these gels due to extract protein, preincubation of the extract with ATP, NADPH and iron appeared to clear the extract protein. Preliminary experiments indicated that when beef heart catalase was added to the incubation mixtures, loss of protein from the extract or from added purified GS was prevented. The proteolytic effect is greatest when incubations are carried out in a shaking water bath at 37° C and this proteolytic activity is observed in crude extracts, as well as extracts dialyzed against buffer containing DTT. These observations are very qualitative and preliminary at best, and therefore a more quantitative assay is required for characterization of this activity.

Proposed Course of Action

There is still much to be learned about the hydrolysis of glutamine synthetase by lysozyme. Clearly lysozyme-induced proteolysis is different from that observed with subtilisin or trypsin in model systems by Dautry-Varsat et al. (J. Biol. Chem. 254:3124, 1979) and Lei et al. (J. Biol. Chem. 254:3129, 1979). The most obvious questions which remain concern (1) the specificity of lysozyme induced hydrolysis, (2) location of susceptible cleavage sites, (3) the mechanism of the reaction, (4) the possible identification of a peptide product which might be a potent inhibitor, and (5) the nature of ATP stimulation. The most important question to resolve is the nature of ATP stimulation, since other investigators have reported variable ATP stimulation of degradation of β-galactosidase X-90 fragment in lysozyme-prepared E. coli extracts (Murakami et al., Fed. Proc. 36:724, 1977). However, the thrust of this project will be the development of a more rapid quantitative assay for in vitro proteolysis of specific enzymes by catalase-deficient cell-free extracts in order to isolate and characterize an intracellular degradation system. The technique of choice would be the preparation of ^{3}H-labeled or ^{125}I-labeled GS substrate in conjunction with TCA fractionation procedures and autoradiography of polyacrylamide gels for the identification of specific procedures.

Publications

Dautry-Varsat, Cohen, G. N., and Stadtman, E. R.: Some Properties of Escherichia coli Glutamine Synthetase after Limited Proteolysis by Subtilisin, J. Biol. Chem. 254, 3124-3128, 1979.

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PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Protein Structure: Enzyme Action and Control

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

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3.7	3.2	0.5

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

Research in this Section consists of studies on the physical and chemical properties of proteins of biological interest and the roles of ligand binding and protein-protein interactions in enzyme catalysis and regulation.

(1) Interactions of divalent cations, substrates, and inhibitors with glutamine synthetase from Escherichia coli have been studied by calorimetry, ultracentrifugation, equilibrium dialysis, pH, spectral, thermal perturbation, and kinetic techniques. L-Methionine-SR-sulfoximine (a transition state analog) promotes both local and gross conformational differences between unadenylylated and adenylylated enzymes. This ligand also affects Mn^{2+} and adenylyltransferase interactions with glutamine synthetase.

(2) Calorimetric and ultracentrifugal studies of the substrate-promoted conformational transition of aspartate transcarbamoylase from E. coli have been performed. The heats of assembly of this enzyme (C_2R_3) from catalytic chain trimers (C) and regulatory chain dimers (R) have been measured in the absence and presence of a bisubstrate analog.

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Project Description

Objectives

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

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

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

(4) Kinetic and equilibrium studies of protein-metal ion interactions, using glutamine synthetase and dye-metal ion complexes, using the enzyme + Me^{2+} in the presence of pH-indicator dyes, and using the enzyme $\pm$ substrate (or substrate analog) + Me^{2+} and atomic absorption.

(5) To investigate the role of AMP in supporting γ-glutamyl transfer activity of unadenylylated manganese-glutamine synthetase.

(6) To measure the enthalpy change for the substrate-promoted transition of aspartate transcarbamoylase from E. coli, using two calorimetric approaches and ultracentrifugal techniques.

Major Findings

(1) Equilibrium binding studies on the interactions of L-methionine-SR-sulfoximine with unadenylylated and adenylylated glutamine synthetase forms from E. coli. (Investigators: A. Shrake, E. J. Whitley, Jr., and A. Ginsburg). Glutamine synthetase, a strictly regulated enzyme in E. coli, is a dodecamer with twelve catalytic sites. L-Methionine-SR-sulfoximine (a proposed transition state analog of glutamine synthetases) produces tyrosyl residue perturbations when it binds to unadenylylated but not to adenylylated glutamine synthetase from E. coli, as evidenced by UV difference spectra. Binding L-methionine-SR-sulfoximine to complexes of unadenylylated glutamine synthetase (manganese-enzyme, magnesium-enzyme, and ADP-manganese-enzyme) produces red-shifted, UV difference spectra consistent with the burial of approximately one tyrosyl residue per unadenylylated subunit. This may involve primarily the tyrosyl residue at the site of adenylylation because the binding of L-methionine-SR-sulfoximine to the fully adenylylated manganese-enzyme produces a red-shifted spectral perturbation of covalently bound 5'-adenylate groups without concomitant tyrosyl perturbations. Spectrophotometric results indicate also that both L-methionine-S-sulfoximine and L-methionine-R-sulfoximine bind reversibly with about the same affinity to glutamine synthetase and compete for a single subunit site. Hill plots of spectrophotometric titrations of the unadenylylated enzyme with L-methionine-SR-sulfoximine give:

$[S]_{0.5}$ = 46 μM and n_H = 0.9 for manganese-enzyme; $[S]_{0.5}$ = 9 μM and n_H = 1.0 for the ADP-manganese-enzyme complex; $[S]_{0.5}$ = 1.9 mM and n_H = 0.5 for the magnesium-enzyme. For binding L-methionine-SR-sulfoximine to the fully adenylylated manganese-enzyme, $[S]_{0.5}$ = 0.19 mM and n_H = 0.7. The observed saturating difference spectrum of partially adenylylated Mn-enzyme (GS_6^-) with L-methionine-SR-sulfoximine is the same as that calculated by averaging those of unadenylylated and fully adenylylated Mn-enzymes. This is additional evidence that unadenylylated and adenylylated subunits show independent difference spectra on binding L-methionine-SR-sulfoximine. L-Glutamate (but not L-glutamine) also produces a small tyrosyl perturbation when it binds to the unadenylylated Mn-enzyme.

(2) Ultracentrifugation studies. (Investigator: A. Ginsburg). Sedimentation velocity and sedimentation equilibrium techniques have been applied to a number of different problems during this past year. A two-cell sedimentation velocity technique (using schlieren optics) for measuring small changes in the sedimentation coefficient of a macromolecule has been used extensively. Measurement of the concentration dependence of the sedimentation coefficient ($\Delta s/\Delta c$) of a macromolecule is representative of its hydrodynamic behavior and has had several applications. These studies have shown that: (a) A modification of E. coli glutamine synthetase by pyridoxylation (E. J. Whitley, Jr., and A. Ginsburg, J. Biol. Chem. 253, 7017-7025, 1978) does not cause dissociation of the dodecamer or any significant change in shape; (b) In an experiment performed for J. Siedel (J. Siedel and E. Shelton, Arch. Biochem. Biophys. 192, 214-224, 1979) glutamine synthetase of A. vinelandii had the same sedimentation coefficient as had the E. coli enzyme; (c) In experiments with A. Dautry (A. Dautry-Varsat, G. N. Cohen, and E. R. Stadtman, J. Biol. Chem. 254, 3124-3128, 1979) limited proteolysis of E. coli glutamine synthetase with subtilisin resulted in a sedimentation coefficient decrease of less than 9%, indicating that the dodecameric structure of glutamine synthetase is maintained after this treatment; (d) In sedimentation equilibrium studies performed for H. Gadasi and E. Korn (Laboratory of Cell Biology, NHLBI), purified Acanthomyosin I had M_w ~ 154,000; (e) In current experiments for B. A. Hemmings (Laboratory of Biochemistry, NHLBI), the dephospho- and phospho- forms of glutamate dehydrogenase from yeast are being characterized by ultracentrifugation.

For the calorimetric studies on E. coli aspartate transcarbamoylase (ATCase) by A. Shrake in collaboration with Dr. H. K. Schachman described in Section 7, sedimentation velocity techniques were used in order to establish the conditions for performing calorimetric experiments and to check the material from the calorimeter after each run to be certain that the anticipated reaction had occurred. In reconstitution experiments for assembly of ATCase (300,000 M_r), the preparations of catalytic (C) subunits (99,000 M_r) and of regulatory (R) subunits (34,000 M_r), received from the University of California at Berkeley, California, varied in stability. Consequently, it was necessary to establish by ultracentrifugation the molar ratio of regulatory to catalytic chains (using an excess of R-subunits) to be used in each set of assembly experiments. In sedimentation velocity experiments, the

boundaries of free R (~ 2.3 S), free C (5.8 S), and ATCase (C_2R_3; 11.5 S) are well separated. After calorimetric assembly reactions, area measurements from schlieren patterns at C and ATCase radial positions in the centrifuge cell gave the mass of C and ATCase. In our experiments, we obtained 80-100% reconstitution of ATCase based on the limiting amount of C-trimer initially present. We found that the assembly reaction was less favored in the presence of a bisubstrate analog N-(phosphonacetyl)-L-aspartate (PALA), which binds with high affinity to catalytic chains. For calorimetric measurements of the heat of binding PALA to ATCase, the change in sedimentation coefficient (Δs) was determined for each binding reaction. This gives a measure of the conformational transition (T → R) which is promoted by PALA binding to ATCase and for which $(\Delta s/s)_{max}$ = -3.1%. The complete change in the sedimentation coefficient of ATCase occurs when only 4 of the 6 substrate binding sites of ATCase are occupied by PALA (G. J. Howlett and H. K. Schachman, Biochemistry 16, 5077-5083, 1977). After the addition of 0, 2, 4, and 6 equivalents of PALA to ATCase in the calorimeter, measured ($\Delta s/s$) values were in agreement with those published by Howlett and Schachman. Calorimeter reactions of PALA binding to the cataltyic trimer also gave the published sedimentation coefficient change ($\Delta s/s$ = +1.4%). Measurements of ($\Delta s/s$) indicated that the hydrodynamic behavior of ATCase was the same in phosphate and in phosphate-TES buffer mixtures at pH 7.0 so that these two buffers [for which $\Delta(\Delta H')$ of protonation = -4.6 kcal/mole] could be used in the calorimeter to obtain proton release and/or proton uptake data.

Recent sedimentation velocity studies of E. coli glutamine synthetase have shown that L-methionine-SR-sulfoximine (a proposed transition state analog) produces measurable changes in the sedimentation coefficient. In the absence of added ligand, the unadenylylated (GS_I^-) and the fully adenylylated (GS_{IZ}^-) manganese-enzymes have the same sedimentation coefficient, after correcting for the effective molecular weight increase due to covalently bound 5'-adenylate groups. However, the binding of L-methionine-SR-sulfoximine to GS_I^- and to GS_{IZ}^- produces small although different alterations in the hydrodynamic particle shape. The changes in sedimentation coefficients (corrected for buoyant weight changes due to ligand binding) are approximately +0.5% and -0.3% for the L-methionine-SR-sulfoximine binding to the unadenylylated and fully adenylylated manganese-enzymes, respectively. Furthermore, the binding of this analog to the adenylylated enzyme appears to expose sites involved in intermolecular associations. These results together with those from Section 1 indicate that the binding of the L-methionine-SR-sulfoximine at the subunit catalytic site promotes different local and different gross structural changes in unadenylylated and adenylylated glutamine synthetase. This has not been observed previously for the binding of any other ligand to this enzyme.

(3) Kinetic and equilibrium studies on glutamine synthetase-Mn^{2+} interactions. (Investigators: J. B. Hunt and A. Ginsburg). Bromothymol blue and metal binding dyes (xylenol orange and fluorescein complexone), together with atomic absorption, have been used to study the binding of Mn^{2+} to the structural (n_1) and nucleotide (n_2) sites of E. coli glutamine synthetase. During the slow conformational change ($t_{1/2}$ ≃ 4 min at 15° C) produced by the binding

of Mn^{2+} to n_1 sites, the binding constant for Mn^{2+} increases from 1 X 10^5 M^{-1} to 2 X 10^6 M^{-1}. This gives an estimate of $\Delta G' \simeq$ 21 kcal (mole enzyme)$^{-1}$ for the conformational change promoted by Mn^{2+} binding to glutamine synthetase. The proton released from glutamine synthetase, which is synchronous with the conformational change, is shown to be the result of a pK_a change rather than direct replacement by Mn^{2+}. The release of Mn^{2+} from n_1 sites of the enzyme has a half-time of ~ 1 sec at 15° C. Equilibrium binding studies show that L-methionine sulfoximine (a transition state analog) causes a 30-fold enhancement in the affinity of n_1 sites for Mn^{2+}, while not affecting the affinity of n_2 Mn^{2+} sites. In contrast, L-glutamine increases 100-fold the affinity of n_2 sites for Mn^{2+} without affecting Mn^{2+} binding to n_1 sites. Mn^{2+} binding to both n_1 and n_2 sites is enhanced ~ 8-fold by L-glutamate. These studies provide additional evidence that bound L-methionine-SR-sulfoximine or L-glutamate (but not L-glutamine) interacts directly with Mn^{2+} bound to high-affinity (n_1) sites of the enzyme. For Mn^{2+} binding studies, an improved method was developed for preparing metal ion-free protein and effector solutions.

(4) Studies on the adenylyltransferase-catalyzed adenylylation of glutamine synthetase. (Investigator: A. Ginsburg). Studies on the effects of L-methionine-SR-sulfoximine on the adenylylation reaction catalyzed by glutamine synthetase adenylyltransferase show that the tight binding of L-methionine sulfoximine phosphate + ADP at the catalytic sites of glutamine synthetase completely blocks adenylylation of tyrosyl residues at adenylylation sites of glutamine synthetase. It was observed also in these studies that both L-methionine-SR-sulfoximine and L-2-amino-3-ureido propionic acid (albizzin; an analog of L-glutamine) act as allosteric activators of the adenylyltransferase; the L-glutamine analog 2-amino-4-oxo-5-chloropentanoic acid did not activate or interfere with L-glutamine-activation of the adenylyltransferase in the adenylylation reaction.

(5) Studies of a novel reaction catalyzed by E. coli glutamine synthetase: AMP-dependent synthesis of pyrophosphate and L-glutamate from orthophosphate and L-glutamine. (Investigators: E. J. Whitley, Jr., and A. Ginsburg). AMP (substituted for the nonconsumable substrate ADP) supports the γ-glutamyl transfer reaction:

$$\text{L-Glutamine} + NH_2OH \xrightarrow[(AMP)]{(Mn^{2+},\ P_i)} \gamma\text{-Glutamylhydroxamate} + NH_4^+ \qquad (1)$$

catalyzed by unadenylylated glutamine synthetase from E. coli (J. B. Hunt, P. Z. Smyrniotis, A. Ginsburg, and E. R. Stadtman, Arch. Biochem. Biophys. 166, 102-124, 1975). In current studies of this reaction, it was discovered that AMP supports the synthesis of PP_i from P_i in the following reaction:

$$\text{L-Glutamine} + 2\ P_i \xrightarrow{(Mn^{2+},\ AMP)} \text{L-Glutamate} + PP_i + NH_4^+ \qquad (2)$$

catalyzed by the unadenylylated enzyme (GS_I^-). The fully adenylylated enzyme

(GS_{I2}) does not catalyze reactions 1 and 2. However, reaction 2 is related to the reverse biosynthetic reaction:

$$\underline{L}\text{-Glutamine} + ADP + P_i \xrightarrow[(\text{or } Mn\text{-}GS_{I2})]{(Mg\text{- or } Mn\text{-}GS_{I})} \underline{L}\text{-Glutamate} + ATP + NH_4^+ \quad (3)$$

which is catalyzed by both the unadenylylated Mg- or Mn-enzyme and the adenylylated Mn-enzyme (M. D. Denton and A. Ginbsburg, Biochemistry 9, 617-632, 1970). In order to identify products and to further characterize these reactions, reactions 2 and 3 were run at pH 7.2 and 30° C in the presence of 1 mM $MnCl_2$ (or 50 mM $MgCl_2$) and L-glutamine (10 mM) with ADP + $^{32}P_i$, AMP + $^{32}P_i$, or [^{32}P]AMP + P_i. Products were identified by charcoal adsorption, thin layer chromatography on PEI-cellulose with autoradiography, and chromatography on Dowex-1 (Cl^--form) with scintillation counting of effluent fractions. With ADP + $^{32}P_i$ in the absence of NH_2OH or ammonia, [^{32}P]ATP was formed in reaction 3 with the unadenylylated Mg- or Mn-enzyme. A high ratio of AMP to ADP or the presence of NH_2OH or NH_4^+ completely inhibits ATP formation in reaction 3. When AMP or [^{32}P]AMP is substituted for ADP in the absence of NH_2OH (reaction 2), AMP remains unchanged and $^{32}PP_i$ is synthesized from $^{32}P_i$. Studies with $^{32}P_i$ and [^{14}C]glutamine showed that the stoichiometry of reaction 2 is as written; i.e., pyrophosphate bond synthesis is coupled to the hydrolysis of the amide bond of L-glutamine. Hydroxylamine or ammonia completely blocks the PP_i formation of reaction 2; hydroxylamine produces reaction 1. ADP competes with the synthesis of PP_i since with the unadenylylated Mn-enzyme, there is competition between reactions 2 and 3. With the unadenylylated Mn-enzyme at pH 7.2 and 30° C in the presence of L-glutamine, AMP, and P_i (12 mM of each), the ratio of AMP-dependent PP_i formation in reaction 2 to AMP-supported transferase activity (with NH_2OH in reaction 1) ≃ 0.01/2.3 or ~ 0.4%. For the same enzyme complex at pH 7.2 and 30° C, the ratio of AMP- to ADP-supported transferase activity in reaction 1 is ~ 2.3/4.0.

Earlier studies in this laboratory indicated that AMP is an allosteric inhibitor of glutamine synthetase. In fact, more recent results show that both [^{32}P]AMP and [^{14}C]ADP can bind simultaneously to the manganese-enzyme subunit. Because AMP is unchanged in reaction 2, AMP could promote the synthesis of PP_i from P_i bound to the γ,β-positions of the ATP binding site by itself binding either to the subunit catalytic site or to the subunit allosteric site. This has not been resolved, but in the presence of ADP, AMP, and P_i, the binding of P_i is antagonistic to that of AMP at the subunit allosteric site, whereas the binding of P_i and ADP at the subunit catalytic site is strongly synergistic. The effect of P_i on the binding of AMP to the enzyme in the absence of ADP is being determined now.

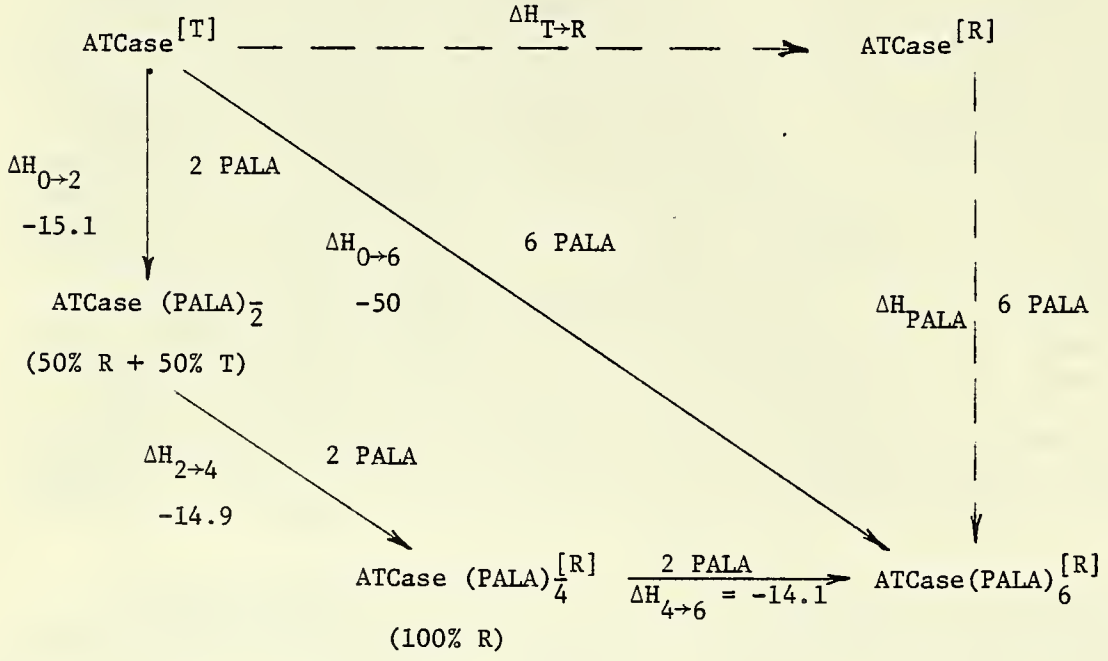
(6) Studies of a reversible thermal transition in glutamine synthetase. (Investigator: A. Shrake). A reversible thermal transition in E. coli glutamine synthetase in the presence of saturating Mn^{2+} at pH 7 with a transition midpoint (melting temperature or T_m) of 51° C was detected by

measurement of the UV temperature difference spectrum. This temperature difference spectrum for the thermal transition is compatible with the exposure of approximately 1 tryptophan side chain per enzyme subunit. The T_m of the thermal transition appears independent of the divalent cation present (Mn^{2+} or Mg^{2+}) and independent of the state of adenylylation of the enzyme. However, substrates (ADP, L-glutamine, and ADP + L-glutamine) and a transition state analog (L-methionine-SR-sulfoximine) increased T_m. Subsaturating levels of Mn^{2+} or Mg^{2+} thermally destabilized the enzyme (T_m decreased). The divalent cation-free form of the unadenylylated protein showed no thermal transition, indicating that the tryptophan burial observed on adding divalent cation to this enzyme probably involves the same tryptophan that becomes exposed during the thermal transition of the Mn- or Mg-enzyme. Nonlinearity of the Arrhenius plot (concave downward) above 50° C in the pH 7.0 γ-glutamyl transfer activity suggests that the observed thermal perturbation is related to a reversible loss of catalytic activity. Immunoprecipitation studies (in collaboration with R. J. Hohman) using purified anti-AMP specific antibodies showed that the glutamine synthetase dodecamer does not dissociate into subunits during the reversible thermal transition. Immunotitrations of unadenylylated enzyme, fully adenylylated enzyme, a mixture of unadenylylated and fully adenylylated enzymes (equivalent to an average state of adenylylation of 6, i.e., equivalent to GS_6^-), and a partially adenylylated enzyme preparation (GS_6^-) (primarily composed of hybrid dodecamers, i.e., those containing both types of subunits) were performed on the above proteins in the presence of Mn^{2+} before and after annealing (at a temperature at which the thermal transiton is complete --- 60° C for 10 minutes). Annealing had no effect indicating that no subunit interchange had occurred with the mixutre of unadenylylated and adenylylated Mn-enzymes. The temperature difference spectrum below the region of the thermal transition (< 30° C) will be used to determine the number of exposed tryptophan and the number of exposed tyrosine side chains in the various enzymes and enzyme complexes. The nature of the temperature-induced unfolding (local vs. temperature-induced macromolecular shape changes) will be investigated by viscometry, ultracentrifugation, and CD techniques.

(7) Calorimetric studies with E. coli aspartate transcarbamoylase. (Investigators: A. Shrake, A. Ginsburg, and H. K. Schachman, Fogarty Scholar 1977-78). The purpose of these studies is to obtain estimates of the enthalpy change ($\Delta H_{T \rightarrow R}$) for the substrate-promoted transition (T → R) of aspartate transcarbamoylase (ATCase) from E. coli, which is produced by binding the high affinity, bisubstrate analog N-(phosphonacetyl)-L-aspartate (PALA); the initial state (T-state) by definition has a low affinity for substrate, whereas the final conformational state (R-state) has a high affinity for substrate. Each ATCase molecule has 6 catalytic sites (1 per catalytic chain). The T → R conformational transition precedes that of PALA binding such that binding 2 equivalents of PALA per ATCase (0 → 2 equivalent PALA per ATCase) promotes 0.5 of the T → R transition. The T → R transition is complete when PALA occupies only 4 or the 6 catalytic sites on ATCase. Thus, the difference between ΔH' for binding the last two equivalents of PALA and either the first two equivalents or all six equivalents gives estimates of $\Delta H_{T \rightarrow R}$ (Methods 1a and 1b, respectively). The intrinsic heat of saturating the

enzyme with PALA without the heat for the T → R transition ΔH_{PALA}, is also obtained. In addition, heats of assembly of ATCase (C_2R_3) from isolated catalytic trimers (C) and regulatory dimers (R) in the absence and presence of PALA (ΔH_1 and ΔH_3, respectively) were determined; these values in conjunction with ΔH_{PALA} and the heat for saturating free catalytic trimer with PALA, ΔH_2, permit another estimate of $\Delta H_{T\to R}$ (Method 2). All calorimetric studies were monitored using the ultracentrifuge (Section 2). This gives a measure of the extent of the PALA promoted conformational transition in ATCase and of the extent of C-trimer incorporation into ATCase in the assembly experiments. All measurements were at 30° C in 40 mM K-PO_4 at pH 7.0 unless otherwise stated. All heats are in kcal/mol ATCase (C_2R_3) or in kcal/mol C.

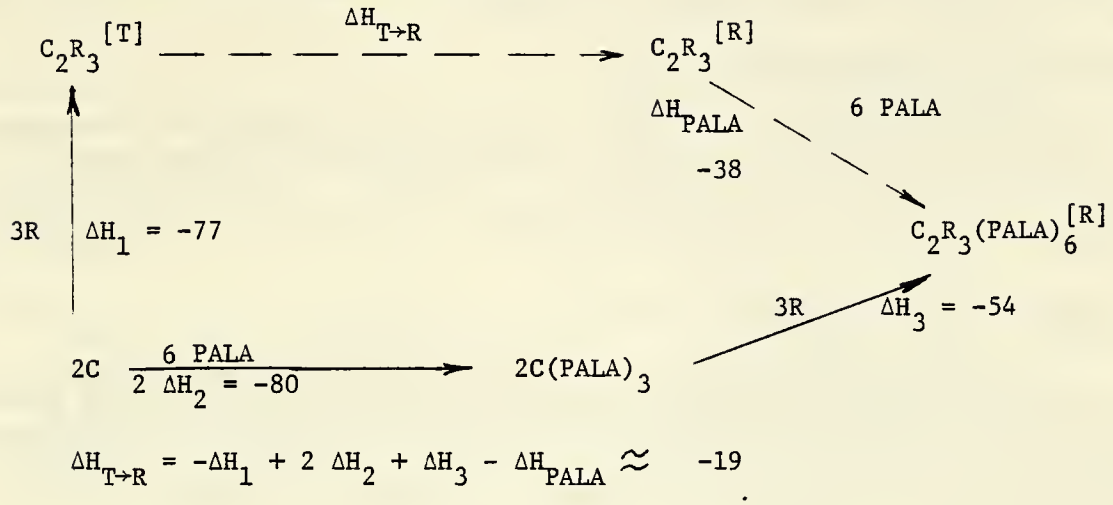
(1) $\Delta H_{T\to R}$ from $\Delta(\Delta H')$ of PALA binding



(1a) $\Delta H_{T\to R} = 2\ (\Delta H_{0\to 2} - \Delta H_{4\to 6}) \approx -2$

(1b) $\Delta H_{T\to R} = (\Delta H_{0\to 6}) - 3\ (\Delta H_{4\to 6}) \approx -8$

(2) $\Delta H_{T\rightarrow R}$ from heats of assembly



$\Delta H_{T\rightarrow R} = -\Delta H_1 + 2\ \Delta H_2 + \Delta H_3 - \Delta H_{PALA} \approx -19$

Experiments using methods 1a and 1b in a buffer with a different heat of protonation (100 mM TES-40 mM K-PO_4 of pH 7.0) permitted an estimate of the proton uptake or release associated with $\Delta H_{T\rightarrow R}$ and ΔH_{PALA}.

Method	$\Delta H_{T\rightarrow R}$	(Attendant equiv H^+ uptake per mol ATCase)	ΔH_{PALA}	(Attendant equiv H^+ release per mol ATCase)
1a	-2	(0.8)	-40	(1.)
1b	-8	(~ 2)	-36	(1.)
2	-19			

From the above calorimetric and ultracentrifuge measurements, we conclude the following: (1) The most accurate estimate of $\Delta H_{T\rightarrow R}$ is probably that from method 1a considering experimental errors involved. Thus, $\Delta H_{T\rightarrow R}$ is negative and small with an attendant proton uptake of ~ 1 equivalent H^+/mol ATCase. The proton release associated with the intrinsic binding of PALA (ΔH_{PALA}) is ~ 1 equivalent H^+/mol ATCase; thus, the net proton effect associated with saturating ATCase with PALA ($\Delta H_{0\rightarrow 6}$) in phosphate buffer is approximately zero. (2) $\Delta S_{T\rightarrow R}$ is negative and small [~ -20 cal/(deg mol)] since $\Delta G_{T\rightarrow R}$ = 3.3 kcal/mol ATCase (G. J. Howlett, M. N. Blackburn,

J. G. Compton, and H. K. Schachman, Biochemistry 16, 5091-5099, 1977). (3) Assembly of C_2R_3 from C and R is less favorable in the presence of PALA. The heat of assembly (in kcal/mol c:r contact) of the unliganded T-state is ~ -13 and of the PALA-liganded R-state is ~ -9.

Significance to Bio-Medical Research

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

Proposed Course of Research

(1) To study conformational and stabilization changes of a protein macromolecule effected through the specific binding of small molecules and the relationship of such effects to enzyme catalysis and regulation. Ultracentrifugation, microcalorimetry, spectral, 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. The interactions between glutamine synthetase and the resolved S- and R- isomers of L-methionine-SR-sulfoximine will be investigated by calorimetry and other physical chemical techniques. Protein conformation changes produced by the binding of L-glutamate and of ADP + P_i to glutamine synthetase will be investigated.

(3) Thermal transitions of glutamine synthetase will be explored further, using activity assay, spectral, viscometry, ultracentrifugation, and circular dichroism techniques.

(4) Kinetic and equilibrium studies of the interactions between Mn^{2+} and glutamine synthetase will be completed. For this purpose, the commercial metal ion binding dyes (xylenol orange and fluorescein complexone) will be purified.

(5) The newly discovered AMP-supported synthesis of PP_i and L-glutamate from P_i and L-glutamine catalyzed by unadenylylated manganese-glutamine synthetase will be investigated in the reverse direction (biosynthesis of L-glutamine). The binding of AMP and P_i and of ADP and P_i to the manganese-enzyme will be studied also.

(6) Calorimetric and ultracentrifugal studies are being performed (in collaboration with H. K. Schachman, University of California, Berkeley, California) with aspartate transcarbamoylase (ATCase) from E. coli and its

catalytic and regulatory subunits to obtain: the $\Delta H'$ value of the conformational transition ($T \rightarrow R$) promoted by substrate binding to the enzyme and the $\Delta H'$ values for the formation of the contact domains of catalytic and regulatory chains in the native enzyme in the T and R states. For additional information on the thermodynamics of assembly and ligand-promoted conformational transitions, ATCase (C_2R_3) also will be assembled from regulatory dimer (R) and an incomplete ATCase molecule (C_2R_2) lacking two regulatory chains. The incomplete C_2R_2 will be prepared at the University of California, Berkeley, California.

Publications

Whitley, E. J., Jr., and Ginsburg, A.: A spectral probe near the subunit catalytic site of glutamine synthetase from Escherichia coli. J. Biol. Chem. 253, 7017-7025, 1978.

Powers, D. M., and Ginsburg, A.: Monomeric structure of glutamyl-tRNA synthetase in Escherichia coli. Arch. Biochem. Biophys. 191, 673-679, 1978.

Ginsburg, A., Shrake, A., Hunt, J. B., and Whitley, E. J., Jr.: Conformational differences between unadenylylated and adenylylated E. coli glutamine synthetase on binding L-methionine sulfoximine. J. Supramolecular Structure, Supplement 3, p. 101, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00205-24 LB

PERIOD COVERED
October 1, 1978 to September 30, 1979

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

PI:	Thressa C. Stadtman	Chief, Section on Intermed. Metabolism & Bioenergetics LB NHLBI
OTHER:	Belinda Seto	Staff Fellow (see indiv. report) LB NHLBI
	Gregory Dilworth	Staff Fellow (see invid. report) LB NHLBI
	Chin-San Chen	Visiting Associate (see indiv. report) LB NHLBI
	Sue H. Neece	Part time Employee (see indiv. report) LB NHLBI
	Maris Hartmanis	Visiting Investigator (Swedish support) 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:
1.8	1.5	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)

A ^{75}Se-labeled macromolecule formed by Clostridium sticklandii in the presence of antibiotics that inhibit either protein or DNA-dependent nucleic acid synthesis was shown to be amino acid transfer nucleic acid (tRNA). Stability and compositional studies suggest that ^{75}Se in the labeled tRNA is located in the ring structure of a modified nucleoside. Incorporation of ^{75}Se is not decreased in the presence of large molar excesses of sulfide of cysteine and therefore is highly specific for Se. The ^{75}Se-labeled tRNA is formed at extremely low Se concentrations and does not turn over rapidly. Chromatographic analysis (see C.S. Chen's report) showed three readily separable ^{75}Se-labeled tRNA species to be present. One of these is a prolyl-tRNA and the other two are in the process of being identified. The possible relationships of these selenium modified tRNAs to selenoprotein biosynthesis or to other regulatory processes will be studied.

PHS-6040
(Rev. 10-76)

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 interraction with other protein components of the enzyme complex. Mode of biosynthesis of the selenium-containing moiety, a selenocysteine residue of the selenoprotein.

   b. Purification and characterization of additional components of the glycine reductase complex in order to determine the mechanism of the reaction and the coupled phosphorylation process.

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

   a. Formate dehydrogenases of Methanococcus vannielii and Clostridium sticklandii.

   b. Identification of high molecular weight seleno-iron sulfur protein complex produced by Clostridium kluyveri.

   c. Mechanism of formation of tRNAs modified with selenium and their biochemical roles.

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

Major Findings

(1) To elucidate the mechanism of glycine reduction to acetate and ammonia and the concomitant synthesis of ATP the proteins that make up the glycine reductase complex have been further characterized. Procedures have been worked out for the isolation of the 12,000 dalton selenoprotein A from reaction mixtures containing ^{32}P-orthophosphate in order to determine if it is the phosphorylated intermediate in the process. A thiophosphate ester of a cysteine residue (or perhaps a selenophosphate ester of a selenocysteine) in this protein is a chemically attractive intermediate.

The 200,000 dalton protein B, a carbonyl group protein that reacts with glycine to form a Schiff base, has been shown by Dr. Sue Neece to contain tritium labeled lactate after reduction with ^{3}H-borohydride. This provides evidence that the carbonyl group of the protein is a covalently attached pyruvyl group rather than pyridoxal phosphate. Earlier spectral studies also indicated the absence of pyridoxal phosphate.

Studies on the biosynthesis of selenocysteine residue of selenoprotein A show that ^{75}Se provided in the form of ^{75}Se-selenocysteine is incorporated much more effeciently than $H_2{}^{75}Se$. However, the selenoamino acid is so rapidly cleaved by C. stocklandii that experiments with ^{14}C-labeled or ^{3}H-labeled Se-cysteine are not interpretable. To circumvent this problem selenocysteine containing enzymes in other bacteria that do not rapidly degrade the administered selenocysteine (e.g. the formate dehydrogenase of M. vannielii, the nicotinic acid hydroxylase of Clostridium barkeri are being investigated. A high molecular weight unidentified selenoprotein of Clostridium kluyveri also may be a viable candidate for this study. Since selenocysteine also occurs in the one known mammalian selenoenzyme (glutathione peroxidase) it is especially interesting to identify the biochemical processes that result in the specific occurrence of this unusual amino acid within the polypeptide chains of the various selenoenzymes.

(2) The finding that C. sticklandii and a few related anaerobic bacteria produce species of tRNA that contain selenium indicates yet another type of modification reaction that enables the cell to regulate a biochemical process in a highly specific manner. Whether these selenium containing tRNAs are involved in processes specifically related to the selenoprotein biosynthesis or to other quite different regulatory systems is as yet unknown.

Efficient incorporation of ^{75}Se into the tRNAs of C. sticklandii occurs at Se concentrations more than 10 fold lower than are optimum for selenoprotein A biosynthesis. The tRNA modification reaction is very rapid and is catalyzed by permeabilized cell suspensions and by cells treated with antibiotics that inhibit protein and DNA-dependent nucleic acid biosynthesis. Both $H_2{}^{75}Se$-selenocysteine (0.05 - 1 μM concentrations) are utilized as Se source. Incorporation of Se is not affected by 1000 to 2000 fold molar excesses of H_2S and/or cysteine indicating the highly specific nature of the Se modification reaction. Spectral analysis of the total tRNA fraction of C. sticklandii indicates the presence of 4-thiouracil in amounts similar to that in E. coli tRNA. Cold chase experiments showed that the ^{75}Se labeled tRNA fraction is relatively stable in the cell and does not contribute Se for the synthesis of selenoprotein A.

Proposed Course of Research

(1) Studies in progress will be continued to determine the origin of the selenocysteine residue in the various bacterial selenoproteins. Location of the selenocysteine residue in selenoprotein A in reference to the two cysteine residues will be pursued. As discussed above the possible role of one or more of these residues in the phosphate esterification step of the catalytic reaction (glycine reduction to acetate) will be investigated in more detail.

(2) The selenium modified tRNAs of C. sticklandii and other anaerobic bacterial will be subjected to chemical analysis to identify the labeled nucleoside(s). The labeled tRNA fraction has been cleaved with ribonuclease and with alkali and the ^{75}Se-nucleotide fragments separated by ion exchange and thin layer chromatography. Similar studies will be carried out on a larger scale to obtain material sufficient for chemical identification. The mechanism of the tRNA modification reaction will be investigated in the in vitro enzyme system.

(3) In collaboration with S. Yamazaki and L. Tsai the importance of the 5-deazaflavin dependent formate-$NADP^+$ oxidoreductase system of M. vannielii for methane biosynthesis will be examined. Attempts will be made to see if the reduced 5-deazaflavin is used directly as reductant for the terminal reaction step in which a methyl group is reduced to methane. An alternative possibility that NADPH is a necessary intermediate will be tested. Current interest in biological formation of hydrocarbons makes these studies at the enzyme level of timely significance.

Honors

Secretary of the American Society of Biological Chemists (2nd year of 3 year term).

Recipient of Hillebrand Award (March 1979) from the Chemical Society of Washington, D. C.

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

Plenary lecturer at Third International Symposium on Organo-Selenium and Tellurium Chemistry, Metz, France, July 1979.

Member of Planning Committee for Second International Symposium on Selenium in Biology and Medicine (Spring 1980).

Publications

T. C. Stadtman. Biological Function of Selenium. Advances in Enzymology 48, 1-28, (1979) John Wiley & Wons, A. Meister, Ed.

Hidehiko Tanaka and T. C. Stadtman. Selenium-dependent Clostridial Glycine Reductase. Purification and Characterization of the two membrane-associated Protein Components. J. Biol. Chem. 254, 447-452 (1979).

Jay B. Jones, Gregory L. Dilworth and T. C. Stadtman. Occurrence of Selenocysteine in the Selenium-dependent Formate Dehydrogenase of Methanococcus vannielii, Archiv. Biochem. Biophys. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00206-20 LB

PERIOD COVERED
October 1, 1978 to September 30, 1979

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 Chemist LB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	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)

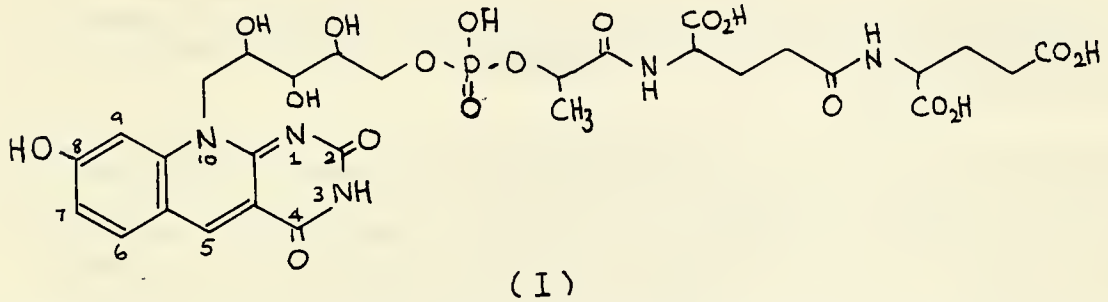
In order to understand the biochemical function of a 5-deazaflavin cofactor from Methanococcus vannielii, two analogs of 8-hydroxy-5-deazaflavin were synthesized and their chemical and spectroscopic properties were studied.

50

PHS-6040
(Rev. 10-76)

Project Objectives

Extracts of Methanococcus vannielii contain a novel 5-deazaflavin cofactor (Jones and Stadtman) which is most likely identical with the cofactor F_{420}(I) (Wolfe et al.) from other methane-producing bacteria.

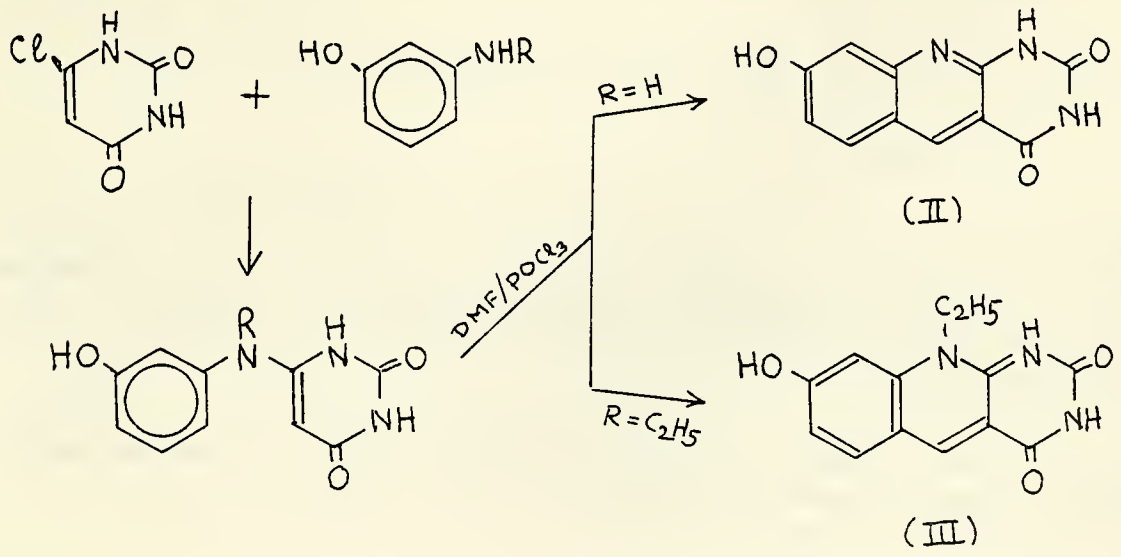


(I)

This 5-deazaflavin cofactor acts as an electron-transport agent in a $NADP^+$-reductase coupled formate dehydrogenase system (Jones and Stadtman). The distinctive character of this 5-deazaflavin is the presence of a hydroxyl group at position 8 giving rise to a number of tautomeric forms. The parent heterocyclic system, 8-hydroxy-3,4-dihydro-2,4-dioxopyrimido[4,5-b]quinoline (II), is hitherto unknown, thus, studies of its chemical behavior would be of importance to the understanding of its biochemical function.

Major Findings

Synthesis of (II) and its 10-ethyl derivative (III) were accomplished as outlined in the Scheme.



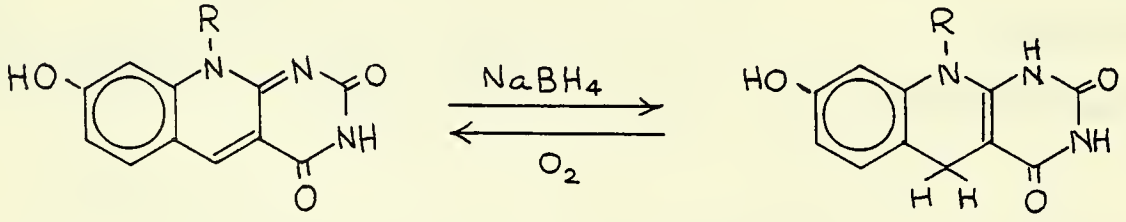
(II)

(III)

In this synthesis, the final cyclization step could occur either ortho or para to the hydroxyl group. The NMR data of (II) and (III) showed only one ortho coupling in the aromatic ring protons demonstrating that only the para ring closure took place. The similarity of the NMR and UV spectroscopic properties to those of cofactor (I) gave support to the original assignment of the structure (I) for the cofactor.

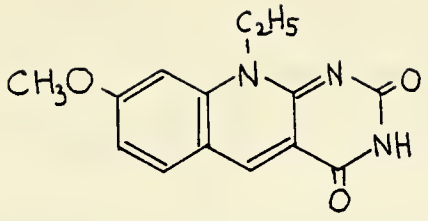
Compounds (II) and (III), which represent the most simple analogs of the natural 5-deazaflavin cofactor were found to be active enzymatically when tested with the $NADP^+$-reductase from M. vannielii, although at lower rates than the natural cofactor.

Reduction of (II) and (III) by sodium borohydride in sodium carbonate solution gave the corresponding dihydro compounds. The dihydro compounds were characterized by their mass spectra (an increase of two mass units in the molecular ion) and their UV absorption (a shift of the λ max at 400 nm region to one at Ca 320 nm). The structures of the dihydro compounds were revealed by the NMR spectra: the low field resonance, assigned to the C_5-H in (II) and (III), vanished upon reduction and was accompanied by the appearance of resonance at δ ~ 3.6 ppm. This established conclusively that the site of reduction took place at C-5, and that the reaction could be represented as follows:

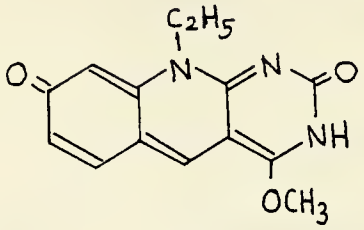


This mode of reaction is probably true for the enzymatic reaction involving the $NADP^+$-reductase from M. vannielii and is in agreement with that observed in other 5-deazaflavin systems. The dihydro compounds upon exposure to air slowly reverted to the oxidized forms, (II) and (III), as indicated by their UV and NMR spectra.

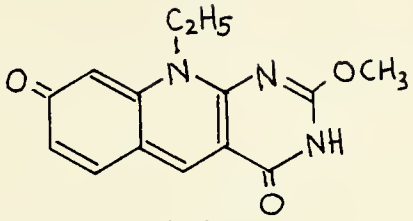
Alkylation of (III) with excess methyl iodide gave mainly a monomethyl and a very small amount of a dimethyl derivative. The NMR of the monomethyl compound showed the methyl resonance at δ 4.0 ppm, suggesting probably an O-methyl instead of N-methyl structure.



(A)



(B)



(C)

However, there are three possible O-methyl structures, (A), (B), and (C), and the present spectroscopic data do not allow unambiguous assignment.

A monoacetyl compound was also obtained from (III) by reaction with acetic anhydride. The UV of the acetyl derivative in methanol resembled that of the monomethyl derivative suggesting similarity in the methylation and acetylation reactions. Due to the unusually labile nature of the acetyl group, no spectroscopic data in different pH could be obtained -- trace of alkali in methanol was sufficient to cleave the acetyl function. Elucidation of the alkylation and acylation reactions must await further studies.

Proposed Course of Action

(1) Chemical behaviors of the 5-deazaflavin cofactor and its synthetic analogs will be pursued.

(2) The stereochemical course of the 5-deazaflavin-dependent $NADP^+$-reductase in M. vanniel ii will be investigated.

Publications

Tsai, L., Silverton, J. V., and Lingh, H. T.: Reinvestigation of the Reaction of Coumalyl Chloride with Ammonia and Amines. α-Aminomethylene glutaconic Anhydride: Structure and Properties, J. Org. Chem. 43, 4415, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00208-06 LB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Structural Characterization of the Proline Reductase Complex

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

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

COOPERATING UNITS (if any)
None

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

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

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

Evidence is presented for an ester bond linkage of the N-terminal pyruvate containing peptide of proline reductase to the remainder of the protein. The evidence is obtained from experiments in which proline reductase is subjected to one of the following treatments: (1) $LiBH_4$ reduction (2) short term hydrolysis with 0.1 N NaOH (3) 25% NH_2OH hydrolysis. The same cleavage site on proline reductase is involved in the three different conditions.

The iron protein, an electron carrier of the proline reductase complex, is a glycoprotein.

PHS-6040
(Rev. 10-76)

Project Description: Project No. Z01 HL 00208-06 LB

Objectives: (1) To characterize the pyruvate containing peptide and its linkage to proline reductase, (2) to develop an alternative enzyme assay for proline reductase, (3) to study the nature of the carbohydrate moieties in the iron protein.

Major Findings:

(1) A pyruvate containing peptide can be isolated from proline reductase by subjecting the enzyme to any one of the following treatments: (a) 0.1 N NaOH hydrolysis at 100° for 10 min; (b) 1 M $LiBH_4$ reduction at 4° for 24 hr; (c) 25% NH_2OH, pH 7 at 25° for 2 hr. The peptide obtained by these procedures appears to be identical based on amino acid composition and C-terminal analyses. These results indicate that the same site of cleavage is involved in all three procedures. Moreover, the alkaline lability and the reactivity with $LiBH_4$ suggest that cleavage occurs at an ester linkage.

(2) A rapid, fluorometric assay for proline reductase is established. The assay involves the addition of o-phthalaldehyde to the reaction mixture for the formation of a fluorescent derivative of δ-aminovalerate, the enzyme reaction product. The fluorogenic reagent reacts specifically with primary amines and therefore proline, the substrate, does not interfere with the assay.

(3) The iron protein, an electron transport component of the proline reductase complex probably contains carbohydrates based on a positive test with the periodate-arsenite-Schiff reagent. Acid hydrolysates of the iron protein can be acetylated and reduced by acetic anhydride and sodium borohydride respectively. Acetyl alditols can be detected by gas chromatography. The identification of the alditols are in progress.

Publications:

Seto, Belinda. Proline reductase: a sensitive fluorometric assay with o-phthalaldehyde. Anal. Biochem., 95: 44-47, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00211-06 LB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Mechanisms of Cellular Regulation - Properties of Regulatory Enzymes

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

P.I.:	E. R. Stadtman	Chief, Laboratory of Biochemistry	LB NHLBI
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COOPERATING UNITS (if any)
None

LAB/BRANCH
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SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.7	3.3	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) Treatment of Escherichia coli glutamine synthetase (GS) with various cross-linking reagents led to the generation of at least 10 different molecular weight species when the treated enzyme is subjected to sodium dodenyl sulfate disc gel chromatography.

(2) From measurements of the rapid quenching of tryptophan fluorescence associated with the binding of anti-AMP-antibodies with adenylylated GS subunits, it was found that the primary binding of antibodies to adenylylated subunits is independent of the fraction of adenylylated subunits per molecule. However, measurements of the slow change in light scattering which follows the primary interaction show that lattice formation is dependent on both the number of adenylylated subunits per molecule and on the total concentration of adenylylated subunits.

(3) Dissociation and reassociation of mixtures of unadenylylated and fully adenylylated GS molecules leads to hybrid molecular forms which exhibit immunoprecipitation patterns that are similar to those of partially adenylylated native GS preparations.

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Project Description

Objectives

Previous studies in this laboratory showed that anti-AMP antibodies obtained by immunizing sheep with chemically adenylylated bovine serum albumin react with adenylylated subunits of Escherichia coli glutamine synthetase (GS), but not with unadenylylated subunits. It was further shown that GS molecules in which all 12 subunits are adenylylated are quantitatively precipitated by the anti-AMP-antibodies, whereas only a fraction of GS molecules composed of both adenylylated and unadenylylated subunits (hybrid molecules) are precipitated. The present study was undertaken in order to: (1) determine if the failure to precipitate all partially adenylylated GS molecules is due to lack of reaction with antibodies or to constraints on lattice formation, (2) determine if hybrid molecules generated by dissociation and dissociation of mixtures of fully adenylylated and unadenylylated enzyme preparations are immunochemically similar to naturally occurring hybrid enzyme preparations, (3) find conditions by which the anti-AMP-antibody complexes could be stabilized by covalent cross-linking reagents so that they can be examined by electron microscopy tc ascertain the mechanism of the antibody-enzyme interactions.

Major Findings

(1) Effect of Adenylylation State on the Binding of Anti-AMP-Antibodies with Adenylylated Subunits of Glutamine Synthetase. Since the interaction of anti-AMP-antibodies with adenylylated subunits leads to quenching of the intrinsic tryptophan fluorescence of one or both reactants, the interaction can be quantitated by stopped-flow fluorescence techniques. Results of such measurements show that the amplitude of the fluorescence change, which is complete in 10 seconds, is proportional to the number of adenylylated subunits present, and is independent of the state of adenylylation of the enzyme. This shows that the failure to precipitate all adenylylated subunits in partially adenylylated enzyme preparations is not due to a failure of all adenylylated subunits to react with antibodies. This conclusion is supported further by the demonstration that the nonprecipitable fraction of adenylylated enzyme is precipitated by the addition of rabbit anti-sheep IgG antibodies.

(2) Immunoprecipitation of Synthetic Hybrid GS Molecules. In earlier studies, it was found that dissociation of mixtures of fully adenylylated and unadenylylated GS preparations by incubation with 7 M urea, followed by dialysis to remove the urea, led to reassociation of the adenylylated and unadenylylated subunits into dodecameric aggregates with stability characteristics similar to those of partially adenylylated natural GS preparation, and distinctly different from the original mixtures of adenylylated and unadenylylated enzyme forms. This was interpreted to indicate that the partially adenylylated GS prepared by dissociation-reassociation of GS mixtures and natural enzyme preparations were composed of hybrid molecules containing both adenylylated and unadenylylated subunits, and that heterologous subunit interactions influenced the stability of the dodecameric aggregate. This view is now supported

by the finding that GS molecules produced by dissociation and reassociation of adenylylated and unadenylylated enzyme mixtures exhibit immunoprecipitability characteristics similar to those of partially adenylylated natural enzyme preparations. In both instances, titration with anti-AMP antibodies precipitates only a fraction of the adenylylated subunits, whereas all adenylylated subunits are precipitated from mixtures of fully adenylylated and unadenylylated enzyme preparations that had not been exposed to the dissociation-reassociation cycle.

(3) Reaction of Glutamine Synthetase with Cross-Linking Reagents. Preliminary efforts to visualize the anti-AMP-antibody GS complexes by means of electron microscopy (B. Bowers) have not been highly successful, probably because dissociation of the complexes occurs during preparation of the samples for microscopic examination. In order to optimize conditions for stabilizing the complexes by means of covalent cross-linking reagents, the interactions of undissociated GS with dimethyl superimidate, glutaraldehyde, and carbodiimide has been carried out. With high concentrations of the latter reagent GS is converted to a form that resists dissociation by treatment with dodecylsulfate. In the presence of 1 mM Zn^{2+} and 50 mM $MgCl_2$, treatment of GS with any one of the cross-linking reagents leads to incomplete stabilization of the dodecameric structure. Disc gel electrophoresis of such GS preparations in 0.1% sodium dodecylsulfate leads to the resolution of at least 10 different protein bands of varying molecular weights. When properly characterized, the various protein fractions thus obtained should be useful as molecular weight standards because they represent a graded homologous series of protein molecules that range in molecular weights from 50,000 to 600,000 in 50,000 dalton increments.

Proposed Course of Action

All of the above projects will be extended. Fluorescent and radioactive anti-AMP-antibodies will be prepared to facilitate investigations on the stoichiometry of the antibody-GS reactions with partially adenylylated GS molecules, and on the nature of the nonprecipitable antibody complexes obtained with hybrid GS species. Following treatment of GS anti-AMP-antibody complexes with high concentrations of carbodiimide, the complexes will be subjected to electron microscopic examination to determine if anti-AMP-antibodies can form intramolecular bridges between adjacent subunits within the same hexagonal ring or adjacent subunits in separate hexagonal rings of the dodecameric structure.

Attempts will be made to separate the several molecular species of protein derived from incomplete cross-linked GS preparations by gel filtration or gradient sedimentation techniques. The purified derivatives will be characterized and subjected to electromicroscopic examination to determine the cross-linking patterns.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00212-08 LB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Regulation of Ammonia-Assimilatory Enzymes in Escherichia coli K_{12}

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

P.I.: Mary Anne Berberich Chemist LB NHLBI

COOPERATING UNITS (if any)

None

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INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.4	1.1	0.3

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Genetic and biochemical studies with enterobacteria suggest that the regulation of nitrogen metabolism in these organisms is quite complex. The NH_3-assimilatory enzymes, as well as some amino acid transport systems and catabolic enzymes, appear to be involved in the process. Because of its unique biochemical properties and its important role in nitrogen metabolism, glutamine synthetase is of primary interest. The purpose of this work is to study, via genetic and biochemical techniques, the mechanism by which the synthesis of glutamine synthetase is controlled in Escherichia coli K_{12}. Special attention is focused on identifying the elements of this control and on determining whether they are specific for the regulation of glutamine synthetase or whether they might represent effectors in the regulation of the class of NH_3-assimilatory enzymes within the general scheme for nitrogen control.

PHS-6040
(Rev. 10-76)

Project Description

Objectives

I. Selection of, and genetic studies on, mutants of E. coli K_{12} manifesting alterations in amount or regulation of the NH_3-assimilatory enzymes: glutamine synthetase (GS), glutamate synthase (GAT), and Glutamate dehyrogenase (GDH).

II. Biochemical characterization of these genetic effects.

III. Nutritional studies with wild-type and mutant strains where the levels of these enzymes are measured under a variety of growth conditions in order to ascertain a.) whether a regulatory interrelationship exists for these three enzymes and 2.) whether one or all of them play a regulatory role within the larger framework of nitrogen control.

Major Findings

I. Genetic Studies

a. Selection of mutants - Previously, several spontaneous isolates from an E.coli K_{12} strain, N99, were shown to have reduced doubling times as compared to the parent, in glucose (11mM) - ammonia (100mM) and could utilize 0.1 mM glutamine as N source when grown at 30° on solid media. These independent isolates displayed variable levels of glutamine synthetase (0.3 - 0.6 S.A.) when grown under conditions of excess NH_3 and could further increase the level of GS specific activity in response to limiting NH_3 or addition of D-glutamate. Subsequently, these strains were shown to be resistant to lysis by the E. coli transducing phage p1 and to be able to utilize L-glutamate as sole carbon source in the presence of excess NH_3. Since the highest levels of GS has always been observed in N99 growing rapidly on glutamate nitrogen, it appeared that the faster-growing, glutamate carbon-utilizing strains could provide new insights into the scheme proposed for nitrogen control of GS. In addition, the p1 resistance trait, if shown to be closely related to the growth and "derepression" phonomena, might indicate the involvement of a more general control element in this process.

At 30°C, glutamate carbon-utilizing mutants could be obtained from E.coli K_{12} strains N99 and 3000 at frequencies of 10^{-6} and 10^{-7} respectively, and from E.coli K_{12} Lederberg at a similarly high, but as yet, undetermined, frequency. The suffix, G, indicates the acquisition of the glutamate carbon-utilizing trait. It is followed by the number designating the particular isolate e.g. N99G1, 3000G-1, N99G-2 etc.

On glutamate carbon plates, these colonies elaborate a yellow pigment that does not diffuse into the medium. These colonies are profoundly lac^+ and all tested were resistant to p1 lysis (21/21 derivatives of strain 3000, 30/30 derivatives of strain N99). Spontaneous, glutamate carbon minus, revertants of N99G-1 were isolated and all were shown to be p1 sensitive (3/3). This indicates that the $glutC^+$ mutation is pleiotrophic.

b. Mapping experiments

Because these strains could be lysogenized by Φp_1CAM clr100, the p_1 resistance trait would appear to be due to something other than a receptor deficit. The extremely small burst size following induction of these lysogens suggests some defect limiting the lytic cycle.

Early transduction results do not indicate linkage of $glutC^+$ to either glnA or argG. However, with $argG^+$ as the selected marker on glucose-ammonia plates, 74% (100/136) of the $argG^+$ recombinants were yellow when N99G-1 was donor. No yellow $ArgG^+$ recombinants were obtained with N99 as donor but, in this case, unexpectedly low numbers of recombinants were obtained. The character of the $argG^-$ recipient should probably be investigated in view of the fact that N99G-1 elaborates the yellow pigment only when growing on glutamate carbon. With $glutC^+$ as the selected marker, a preliminary attempt to transfer $glutC^+$ from N99 G-1 to N99 yielded no recombinants. This indicates that the $glutC^+$ phenotype might require a double mutation.

II. Biochemical Characterization of Strains

A. Standardized Conditions - Enzymes were assayed in crude extracts prepared from cells growing under standardized conditions: early logphase (between $5x10^7$ and $5x10^8$ cells/ml) with growth and enzyme levels monitored over a period which included 2-3 mass doublings. In addition to the presence of excess ammonia (N/C = 10/1) the level of GS could be further repressed by addition of glutamine to the medium. Therefore, "repressing" medium=glucose (11mM) mineral salts, Ammonia (20mM), and glutamine (3mM). "Derepressing" medium = glucose (11mM) mineral salts, glutamine (10mM). Specific activity of GS in cells from "repressing" medium is usually .198 for N99 while it varies between .24 and .60 for N99G-1 with the latter showing correspondingly elevated levels of GDH and GAT when GS is elevated. The average specific activity of GS in cells from "derepressing" medium is 1.22 for N99 and 1.28 for N99G-1.

B. Acute type experiments

1. In downshift experiments (from repressing to derepressing media) with N99 G-1, it could be shown that the initial rate of increase in GS level is rapid for about 30 min. when approximately a 6-fold derepression is reached. This is followed by a period of slower increase to approximately 7-fold derepression within 90 min. Comparable experiments with N99 demonstrated that the rate of derepression was constant over a 90 min. period and was

∿7X slower than the initial rate observed with N99G-1. The change in doubling time during this transition was 25 min. for N99G-1 and 92 min. for N99. Although N99G-1 grows approximately 2X faster than N99 under "repressing" conditions, the exaggerated slowdown of growth for N99 on shifting to derepressing conditions points out that the limitation in strain N99 affects both the rate of utilization of glutamine as nitrogen source and the rate of increase in the specific activity of GS. Subsequent experiments of this type determined that the plot of increase in $\bar{n}$ value exactly paralleled that for GS activity with $\bar{n}$ = 3.6 at 0 time and $\bar{n}$ = 8.0 at 65 min. During the period covered by these experiments (approximately 90 min.), the specific activities of GDH and GAT did not change.

2. The level of all 3 NH_3-assimilatory enzymes was consistently lower in cells grown on equimolar (with respect to carbon) amounts of glycerol as compared with glucose as carbon source. D-glutamate added to cells growing in glycerol (66mM), mineral salts, ammonia, (100mM) evoked increases in the specific activities of the 3 NH_3-assimilatory enzymes in both strains N99 and N99G-1. Within a 90 min. period GS increased 2-fold and GDH increased 3-fold. Maximal levels were attained earlier for N99G-1. Whereas, in N99, the rate and fold increase of GAT was identical to that of GS, in N99G-1 the increase in GAT was identical to that of GDH. No effect of D-glutamate addition during ammonia limited growth was observed. Since the presence of glutamine in the growth medium interferes with D-glutamate effect in both permeabilized and unpermeabilized cells, it seems unlikely that D-glutamate acts at the ammonia transport level. The mechanism by which D-glutamate consistently elicits these effects remains obscure at present. However, the fact that all 3 of the NH_3-assimilatory enzymes are affected in the positive direction would appear to be significant and the differences observed between N99 and N99G-1 are consistent with other differences observed during the derepression process.

III. Nutritional Studies

The specific activity of GS is 0.95 in glut C^+ derivatives of *E. coli* K_{12} strains N99 and Lederberg growing in glutamate (20mM), MOPS*, ammonia (10mM). The state of adenylylation is 10 in each case. Increasing the phosphate concentration in the medium from 1 to 100 mM results in an increase in the doubling time (from 71 to 180 min) and a decrease in the level of GS observed (specific activity 0.60) for both strains while the state of adenylylation is not appreciably affected ($\bar{n}$ = 9.5). The changes in doubling time effected by varying the phosphate concentration suggest that the mobilization of glutamate as an energy source is being affected. Whether phosphate is affecting glutamate transport or glutamate catabolism cannot be determined from these experiments but other evidence favors the latter interpretation. The highly adenylylated state of the GS indicates that the major product of glutamate catabolism is probably not α-keto glutarate.

With glutamate as sole source of carbon and nitrogen, N99G-1 grows with a doubling time of 127' in media containing 1mM phosphate and with a doubling time of > 7 hours in media containing 100mM phosphate. Under these two conditions the specific activity of GS is 0.15 and 0.16 respectively. This unexpected result indicates that, in the absence of ammonia nitrogen, these cells are maintaining a minimal level of GS when growing with glutamate as sole carbon source.

These results would suggest that the shift to a more aerobic mode of metabolism can effect an increase in the specific activity of GS. Consequently the specific activity of GS was determined in strains: N99, N99G-1, N99G-1 rev 1, 3000 and 3000G-2, growing on the above media with succinate as carbon source. Although N99G-1 and 3000G-2 grow with markedly decreased doubling times (<1/2 that of parent strains), the specific activity of GS was not appreciably elevated (av. SA = 0.35) although the enzyme was highly adenylylated ($\bar{n}$ = 7.0 - 9.0). It would appear from these studies that some specific product of glutamate catabolism, is either directly responsible for the increase in GS or, induces some fundamental change in the components of an, as yet undefined, attenuation system.

*The MOPS medium of Niedhardt et al. (J. Bact. 119:3p 736, 1979), minus ammonia was used as the mineral salts component of the growth media.

Proposed Course of Action

1.) Genetic analysis of the pleiotrophic glutC$^+$ mutation in order to:

a. define the # of factors involved
b. locate map position/s
c. characterize the gene product/s.
d. discern its role in nitrogen control.

2.) Attempt to define genetic/nutritional conditions which will permit one to determine whether the NH_3-assimilatory enzymes are inducible, derepressible, or regulated by attenuation.

3.) Characterize the compound responsible for the yellow-pigmentation described in Section I.

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PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Control of In vitro Assembly of Microtubules

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

P.I.:	Donita L. Garland	Staff Fellow	LB NHLBI
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COOPERATING UNITS (if any)

None

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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)

A new method for assaying polymerization of tubulin has been developed. The method, which utilizes the colchicine binding properties of tubulin, directly quantitates tubulin incorporation into microtubules.

Under appropriate conditions cAMP and cGMP inhibit the in vitro assembly of microtubules from crude supernatants. cAMP has no effect on the polymierization of purified tubulin. In an effort to determine the mechanism of the cAMP-dependent inhibition of polymerization, the effects of cAMP on protein phosphorylation in the crude supernatant and preparations of purified tubulin have been studied.

ATP, ADP, AMP, and adenosine have inhibitory effects on the polymerization of microtubules from crude supernatants of brain homogenates. The mechanisms of these effects are under study.

PHS-6040
(Rev. 10-76)

Project Description

Objectives

The continuing focus of this study has been on the role of cyclic nucleotides in the regulation of tubulin activity. We previously showed that cAMP in the presence of sodium fluoride inhibited the *in vitro* assembly of microtubules from crude supernatants. The present objectives are to: (1) Further characterize the cAMP-dependent inhibition of polymerization. (2) Determine if cAMP inhibits microtubule assembly through an effect on a cAMP-dependent kinase. (3) Identify the protein that is phosphorylated. (4) Determine how this phosphorylation alters the activity of tubulin.

Major Findings

(1) A new method for assaying polymerization of microtubules was developed. This method utilizes the colchicine binding properties of tubulin, and has the advantages that it is rapid and it directly quantitates the tubulin incorporated into microtubules.

(2) cAMP, in the presence of fluoride, inhibits the polymerization of microtubules from crude supernatants prepared from brain homogenates. The sensitivity to cAMP varies with the preparation. Some preparations require a particulate fraction to obtain the cAMP-dependent inhibition. The ATP-dependent polymerization appears to be more sensitive to inhibition by cAMP. cGMP also inhibits the polymerization of microtubules from crude supernatants.

(3) cAMP, in the presence or absence of fluoride, has no effect on the polymerization of cycle purified tubulin. Fluoride alone causes a slight but consistent stimulation of polymerization.

(4) Once purified, tubulin cannot be modified by crude supernatant in the presence of cAMP and fluoride.

(5) ATP, ADP, and AMP inhibit to varying extents the polymerization of microtubules. The inhibitions by ATP and ADP are overcome by high concentrations of NaF, but not NaCl.

(6) Adenosine also inhibits polymerization. This has only been seen so far when particulate fraction is present. It is possible that the effects of ATP, ADP, and AMP are the result of their metabolism to adenosine.

(7) Initial experiments with the cAMP-dependent protein kinase inhibitor suggest that the cAMP-dependent inhibition of polymerization may involve a phosphorylation or dephosphorylation.

(8) Studies have been started to determine which proteins are phosphorylated in the crude supernatant in the presence of cAMP, fluoride, etc. Initial results have shown dramatic differences in the pattern of phosphorylation in

the presence of the different compounds. The pattern also changes with time.

(9) Adenosine, guanosine, and inosine, but not cytidine, alters the level of phosphorylation in crude supernatants of brain homogenates. The patterns of phosphorylation differ from that found in the presence of cAMP.

Relevance to Medicine

The study of the function and cellular regulation of microtubules has widespread benefits for both medicine and the understanding of cellular function. Microtubules are involved in development of cell shape, in cell motility and mitosis, and in all cases where intracellular particle movement occurs, i.e., axonal transport and secretion. The exact function of microtubules in each of these is still not clear. Microtubules are involved with intramembranal movement of receptors. As a result, microtubules have a role in the control of hormonal activation of adenylate cyclase. Disruption of the microtubule network involved in information transfer from the membrane to the nucleus leads to malignancy. Microtubules are required for mitosis, and thus are a good point for control of malignant cell growth. Likewise, microtubules are important in wound healing and in control of lysosome function. The latter is particularly important in pathogenesis of gout, arthritis, and periodontal disease. Other cases where a defect in control or function of microtubules contributes to disease are: Alzheimer's disease, lung dysfunction, infertility in cases of nonmotile cilia and flagella, and Chediak-Higashi syndrome.

The importance of studying the effects of adenosine is illustrated by its implication in numerous cellular phenomena including cell morphology, cell toxicity, immunosuppression, and neuron function. Adenosine is purported to be a neurotransmitter. It changes intracellular cAMP levels, effects metabolism of fat cells, and has been implicated in the local regulation of coronary blood flow. Aberrations in adenosine metabolism have resulted in diseases such as immunodeficiency disease and hereditary hemolytic anemia. In all of these cases, the exact role of adenosine is not known.

Proposed Course of Action

The following studies will be extended:

(1) Reexamine the role that the nerve ending fraction has on the cAMP dependent inhibition of polymerization.

(2) Identify and purify the proteins that are phosphorylated or dephosphorylated in the presence of cAMP and fluoride in the crude supernatants, and determine how they change the activity of tubulin.

(3) Identify the protein that is phosphorylated by adenosine. Determine if the action of adenosine is on a kinase or phosphatase.

(4) Examine the effect of adenosine on the polymerization of microtubules in more detail.

Publications

Garland, D. L.: Kinetics and Mechanism of Colchicine Binding to Tubulin: Evidence for Ligand-Induced Conformational Change, Biochemistry 17, 4266, 1978.

Garland, D. L.: cAMP Inhibits the In vitro Assembly of Microtubules, Arch. Biochem. Biophys., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00221-03 LB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Control of Glutamate Metabolism

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

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Other:	Earl R. Stadtman	Chief, Laboratory of Biochemistry	LB	NHLBI
	J. Michael Poston	Research Chemist	LB	NHLBI

COOPERATING UNITS (if any)

Dr. Elizabeth Jones, Carnegie-Mellon University, Pittsburgh, Pennsylvania

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NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.1	0.2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Studies on the regulation of the yeast glutamate dehydrogenases (GDH) were continued. Both the active (dephospho-) NADH-GDH and the less active (phospho-) NAD-GDH have been purified to homogeneity and characterized. Two cyclic nucleotide independent protein kinases have been partially purified and shown to be capable of phosphorylating NAD-GDH. Phosphorylation of NAD-GDH in vitro does not result in enzyme inactivation.

The role of yeast proteases in the regulation of NADP-GDH, during carbon starvation, was investigated using mutant strains of Saccharomyces cerevisiae deficient in certain protease activities. Strains lacking protease B activity or with low levels of protease A, B, and carboxypeptidase Y inactivated (degraded) NADP-GDH at a similar rate to that observed with the mild type parent strain.

The processing of procarboxypeptidase Y was investigated in protease deficient yeasts. One of the mutants was found to synthesize procarboxypeptidase, but did not process (activate) the enzyme thus lacking any carboxypeptidase activity.

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Project Description

Glutamic acid plays a central role in nitrogen and carbon metabolism of both eukaryotes and prokaryotes. To understand the metabolism of this vital amino acid, it is necessary to understand the regulation of the enzymes responsible for its synthesis and degradation.

In yeast, the two major enzymes of glutamate metabolism are NAD-dependent glutamate dehydrogenase (NAD-GDH) and the NADP-dependent glutamate dehydrogenase (NADP-GDH). NAD-GDH and NADP-GDH are genetically distinct and perform different physiological functions. The NAD-GDH has a catabolic function, and the NADP-GDH performs a biosynthetic function. The aim of this project is to understand the regulation of these two proteins.

Major Findings

(1) Regulation of NAD-Dependent Glutamate Dehydrogenase

Previous work (see 1978 report) demonstrated that the NAD-GDH from Candida utilis is regulated by phosphorylation-dephosphorylation. The work on this enzyme regulation system has been extended.

a. Characterization of NAD-GDHa (Dephospho-) and NAD-GDHb (Phospho-).

Both GDHa and GDHb have been purified to homogeneity, and a characterization of the two enzyme forms was undertaken. GDHa and GDHb have identical amino acid compositions and appear to be very similar to the NAD-GDH from Neurospora crassa, characterized by Emil Smith and colleagues (J. Biol. Chem. 249, 7922-7928, 1974). The NAD-GDH from N. crassa and C. utilis differ significantly from all other glutamate dehydrogenases because they have a subunit size of 116,000 daltons (cf. 50,000).

Analytical ultracentrifugation studies were initiated. From sucrose density gradient centrifugation, the NAD-GDH was found to have a molecular weight of around 380,000; whereas gel filtration studies indicated that the molecular weight was slightly in excess of 600,000. Thus, the question whether the C. utilis NAD-GDH is a tetamer (as in the case of N. crassa) or a hexamer remains open at this point.

A study of the kinetic properties of the phospho- and dephospho-GDH was undertaken. Early experiments clearly demonstrated a V_{max} difference between the purified GDHa and GDHb. From these studies we hoped to establish whether phosphorylation affected the affinity of the enzyme for its physiological substrates, L-glutamate and NAD. A difference in the apparent K_m for L-glutamate was observed between the two enzyme forms: 34 mM for GDHa and 114 mM for GDHb. Thus, when assayed at subsaturating levels of L-glutamate the difference in specific activity (V_{max}) observed for the two enzyme forms was around 15-fold (cf. 7-fold at almost saturating glutamate). With subsaturating levels of L-glutamate and NAD, the difference in specific activity was almost 40-fold. These experiments would tend to suggest that

the phosphorylated GDH is completely inactive under physiological conditions. The apparent K_m for NAD was 1.0 mM and 1.3 mM for GDHa and GDHb, respectively.

For these studies no attempt was made to fractionate the GDHb to obtain fully phosphorylated enzyme (the native GDHb is probably a hybrid mixture of different phosphorylation states). Therefore, these results should be taken as a minimal difference.

b. Phosphorylation of NAD-GDH by Cyclic Nucleotide Independent Protein Kinase A.

Protein kinase A was partially purified (1000-fold) from C. utilis by chromatography on CM-cellulose, Ultrogel AcA34, Cibacron blue-Agarose, CM-Biogel, and phosphocellulose. SDS-polyacrylamide gel electrophoresis revealed the presence of 3 protein bands in the purified enzyme preparation.

Protein kinase A preferentially phosphorylates acidic proteins such as casein and phosvitin and is totally inactive towards histone, protamine, and bovine serum albumin. It has a molecular weight of around 50,000 (by gel filtration), and a sedimentation coefficient of 4.45s. The apparent K_m values for ATP and Mg^{2+} are 50 μM and 1 mM, respectively, with casein as the protein substrate. Protein kinase A is stimulated (100-300%) by KCl, NaCl, and NH_4Cl at concentrations up to 100 mM with casein as substrate. Addition of NaH_2PO_4, Na_2SO_4, and $CaCl_2$ were found to be inhibitory. cGMP and cAMP had no stimulatory effect upon enzyme activity.

The phosphorylation of GDHa by protein kinase A was extensively studied. Initially, incorporation of phosphate into GDHa was quite low (0.1 to 0.5 mol P / subunit). Acceptor capacity of GDHa was found to vary with different batches of enzyme. The reason for this variability is under further investigation. At pH 8.5, or above, GDH phosphorylation was increased to 1.6 mol P / subunit. However, some of this increase can be attributed to instability of GDH under these conditions; denatured proteins are known to be better substrates for phosphorylation than native proteins. Additionally, the extent of GDHa phosphorylation was shown to depend on the kinase and GDH concentration in the assay system. Under optimal conditions, where both GDH and kinase were stable, about 1 mol P / subunit was incorporated. Several attempts were made to demonstrate loss of GDHa activity accompanied phosphorylation. However, even after the incorporation of 1 mol of P / subunit, no quantitative or qualitative (pH optima change) changes were observed. The phosphate group introduced by protein kinase A could be removed by phosphoprotein phosphatase or Escherichia coli alkaline phosphatase; no activity changes were observed upon dephosphorylation. Thus, it was predicted that protein kinase A phosphorylates GDH at a different site (structural site) to that which is phosphorylated in vivo (regulatory site). This was confirmed by experiments with GDHb, the in vivo phosphorylated enzyme form, where it was demonstrated that it could also serve as a substrate for protein kinase A.

c. Phosphorylation of GDH by Cyclic Nucleotide Independent Protein Kinase B.

A second protein kinase (B) was isolated from C. utilis and partially purified by ammonium sulphate precipitation, DEAE-cellulose chromatography, affinity chromatography on Cibacron blue-Agarose, and phosphocellulose. This kinase had distinctly different properties to protein kinase A, although it also preferred acidic proteins as substrate and could phosphorylate GDH.

The molecular weight, as judged by gel filtration, was around 500,000. It was not affected by cyclic nucleotides, nor inhibited by protein kinase inhibitor protein from rabbit muscle.

Rather surprising, it was found to be stimulated by calmodulin (calcium-dependent regulator protein) from bovine brain. Endogenous phosphorylation of the partially purified kinase was stimulated 2-5-fold by the addition of calmodulin to the reaction mixture. This result is under further investigation.

With GDHa as substrate, up to 0.3 mol of P / subunit was incorporated. Again, no activity changes were observed concomitant with in vitro phosphorylation.

d. Phosphorylation of GDH by Cyclic Nucleotide-Dependent Protein Kinase.

A cyclic nucleotide-dependent activity was identified in C. utilis. Preliminary experiments suggest that it does not phosphorylate GDH.

Also, experiments were conducted using rabbit type I and II cyclic nucleotide-dependent protein kinases. Some phosphorylation of GDH was observed using kinase I and II, however, after experiments using protein kinase inhibitor protein, it was concluded that the observed phosphate incorporation was probably due to contaminating cyclic nucleotide independent protein kinase in the kinase I and II preparations.

(2) Regulation of NADP-Dependent Glutamate Dehydrogenase in Saccharomyces cerevisiae

The immunochemical studies on the proteolytic inactivation of the NADP-GDH were continued. Previously it was demonstrated that loss of NADP-GDH activity during carbon starvation was paralleled by a loss of enzyme protein. In vitro work (had) suggested that yeast protease B was responsible for loss of enzyme activity and protein.

Using mutant S. cerevisiae strains lacking different protease activities, the proteolytic inactivation of NADP-GDH was reexamined. (Yeast mutant strains were obtained from Dr. E. W. Jones, Carnegie Mellon University.)

Strain prb1-1122, totally lacking protease B activity due to an ochre mutation was found to inactivate NADP-GDH (degrade) at a similar rate to that observed with the parent strain. Another strain 20B-12, which carries a pleiotropic mutation that results in deficiences for protease A, B, and C, also inactivates the NADP-GDH quite normally during glucose starvation. From these studies it is concluded that the NADP-GDH is inactivated by an, as yet, uncharacterized protease system.

(3) Biosynthesis of Yeast Carboxypeptidase in Saccharomyces cerevisiae

In wild type yeast, carboxypeptidase (CPY) is synthesized as a zymogen [or procarboxypeptidase (proCPY)] with a molecular weight of about 69,000 daltons, which then becomes cleaved (processed) to form an active enzyme with a molecular weight of 61,000. Based on in vitro experiments both protease A and B were shown to be capable of cleaving proCPY. The processing of CPY was examined in the same mutant strains used in the NADP-GDH studies.

It was found that strain prb1-1122 (lacking protease B activity) processed CPY, whereas strain 20B-12 (the pleiotropic mutant with low levels of protease A, B, and C) was found not to cleave the proCPY. Strain 20B-12, although not able to process proCPY, synthesized normal amounts of this protein. These results show that the loss of CPY activity from 20B-12 is due to the fact that the enzyme is not being activated in vivo, and that protease B is not involved in the processing of proCPY. These results tend to suggest that protease A may be the enzyme responsible for the processing of proCPY.

(4) Isolation of Vitamin B_{12} from Candida utilis

(Work carried out in conjunction with J. Michael Poston, Laboratory of Biochemistry, National Heart, Lung, and Blood Institute.)

Crude extracts prepared from C. utilis were shown to possess a substance that binds to intrinsic factor. Purification of this substance showed it to be a mixture of methylcobalamin and adenosylcobalamin. Two cobalamin-dependent enzyme systems have been found in C. utilis, methylcobalamin-dependent methionine biosynthesis and leucine 2,3-aminomutase. The cobalamin extracted from C. utilis is as effective as authentic adenosylcobalamine in stimulating leucine 2,3-aminomutase.

These results demonstrate that yeasts are not unique in their metabolism, but also synthesize and utilize corrins.

Proposed Course of Action

(1) Experiments on the regulation of NAD-GDH will be continued. It is hoped that an authentic phosphorylation-dephosphorylation system will be

isolated and characterized.

(2) Work on the regulation of protein degradation and processing will be continued, using the mutant strains of yeast, in order to delineate the role of the known yeast proteases in these processes.

Publications

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Hemmings, B.A.: Regulation of Nitrogen Metabolism in Yeast by Proteolysis and Protein Phosphorylation. Cold Spring Harbor Symposium on Yeast Molecular Biology, 1979.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00224-02 LB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Enzyme Mechanism and Kinetic Theory

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

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	Judith Bale	Staff Fellow	LB NHLBI

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H. Milman - Toxicology Branch, NCI, NIH

LAB/BRANCH
Laboratory of Biochemistry

SECTION
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

(1) Detailed analysis of steady-state kinetic studies on the mechanism of action of E. coli alkaline phosphatase has been completed. The flip-flop model is contrary to alternative substrate and product inhibition patterns. The effect of Tris on the k_{cat} of this enzyme can be accounted for by the increased rate of dissociation of the Tris-phosphate product.

(2) A covalent intermediate of asparaginase, the aspartyl-enzyme, can be stabilized at acidic pH and low temperature. It is the first time that the elusive intermediate can be demonstrated.

(3) The kinetic mechanism of asparagine synthetase is shown to be consistent with a hybrid Uni Uni Bi Ter Ping-Pong-Theorell-Chance mechanism with abortive complexes. The "two-site" Ping-Pong mechanism can be ruled out.

(4) Mathematical proofs have been obtained for formulas for the rapid computation of King-Altman patterns. In addition, a method for calculating such patterns in the presence of irreversible steps has been established.

PHS-6040
(Rev. 10-76)

Project Description

Objectives

(1) To gain knowledge of the regulatory and catalytic mechanisms of enzymes.

(2) To develop or improve methods and theory applicable to the study of enzyme mechanisms.

Major Findings

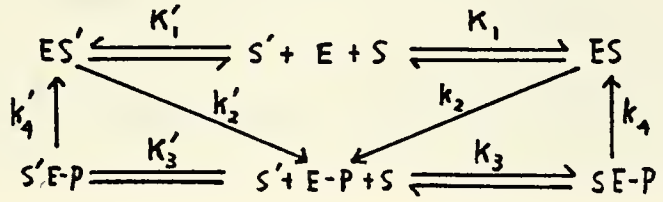
(1) Mechanistic studies on Escherichia coli alkaline phosphatase were undertaken in an effort to understand the biological significance, if any, of extreme negative cooperativity or half-of-the-sites reactivity. The flip-flop model proposed by Lazdunski et al. (Eur. J. Biochem. 20, 124, 1971) to explain the strong anticooperativity of E. coli alkaline phosphatase appears quite attractive because it is based on mechanistic considerations. In this model, dephosphorylation of the phosphoryl enzyme intermediate on one subunit is supposedly facilitated by the binding of substrate to the idle subunit; the second subunit then becomes the site of catalysis to complete the flip-flop cycle.

The use of alternative substrates to test the flip-flop hypothesis has been previously reported. There are some doubts, however, as to whether the alternative substrates employed are sufficiently different in their kinetic parameters to permit a distinction of several kinetic models. Briefly, in the presence of an alternative substrate at constant ratio to the substrate, intersecting double reciprocal plots are predicted for the flip-flop model, whereas parallel plots are expected for the classical mechanism not involving flip-flop. All the alternative substrate studies yielded parallel plots. However, the question can be raised: under what conditions will the flip-flop model also give rise to parallel plots?

Mathematical analysis shows that such a condition is fulfilled by

$$\frac{k_4 K_1}{k_2 K_3} = \frac{k_4' K_1'}{k_2' K_3'}$$

These kinetic parameters correspond to various reaction steps shown in Scheme I.



Scheme I

where S' = alternative substrate and E-P = phosphoryl enzyme intermediate.

It has been known for some time that different substrates for alkaline phosphatase have similar k_{cat}, presumably because they share a common rate-limiting step -- the release of phosphate, k_4 (or k_4'). Thus, it is likely that $k_4 = k_4'$. The magnitude of k_2 and k_2', the rate constants for the phosphorylation reactions, however, can be quite different. If $k_2 \neq k_2'$, the alternative substrate method is still valid. Even if $k_2 = k_2'$, the method remains valid provided $K_1/K_3' \neq K_1'/K_3'$. Consequently, with any two alternative substrates of different K_m's such as CMP (K_m = 2.3 μM) and AMP (K_m = 1.4 μM), the requirement for the flip-flop model to generate parallel reciprocal plots is highly restricted: $k_4 = k_4'$, $k_2 = k_2'$, and $K_1/K_3 = K_1'/K_3'$; that is, the two substrates must have the same phosphorylation rate constants and their affinities for the free E and the E-P complex must maintain a fixed ratio. To impose such restrictions on <u>all</u> the substrates would be quite unreasonable.

When the product inorganic phosphate is present, a pathway between E and E-P in Scheme I appears because Pi is known to combine with alkaline phosphatase to form both noncovalent and covalent complexes. This step is ignored in the flip-flop model of Lazdunski <u>et al.</u>, since only the substrate-facilitated dephosphorylation was thought to be significant. With the E-P $\rightleftharpoons$ E+P step, nonlinear noncompetitive inhibition by Pi is expected for the flip-flop mechanism. The simple linear competitive inhibition observed experimentally, however, is consistent with the non-flip-flop model. For the flip-flop model to generate linear competitive inhibition patterns by Pi, additional conditions are needed: $K_3 \ll S$ and lack of binding of Pi to the ES form. The dissociation constant of S from E-P, K_3, must be very small ($\lesssim$ 0.03 μM) because the lowest concentration of S employed in the experiment was ~ 0.3 μM. Thus, another highly stringent condition must be imposed on the flip-flop model.

The k_{cat}'s for <u>E. coli</u> alkaline phosphatase determined at 10° C and 25° C as a function of Tris buffer concentration showed saturation phenomena. The value of k_{cat} obtained at 0.1 M Tris, 25° C, is 27 sec^{-1}, which agrees with the "off-rate" of Pi, 25 sec^{-1}, determined by Hull <u>et al.</u>, (Biochemistry <u>15</u>, 1547, 1976) using ^{31}P NMR technique. If the flip-flop model is correct, k_{cat} should be the Pi release rate constant from ES. This means that the off-rate of Pi from either free E or ES must be the same magnitude, and the role of S (the S binding to the E-P intermediate) must be restricted to the dephosphorylation step SE-P $\longrightarrow$ SE·P; but transient kinetic studies already demonstrated that the presence of S had no influence on this step. It can be concluded, therefore, that despite the attractiveness of the flip-flop model from a mechanistic standpoint, it is not the mechanism operative in the <u>E. coli</u> alkaline phosphatase system.

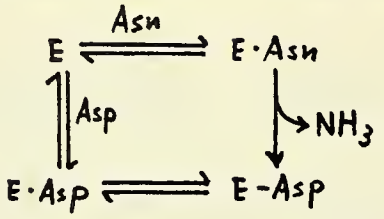
Analysis of the saturation phenomena of k_{cat} as a function of Tris concentration further reveals that the following relationship exists:

$$\frac{1}{k_{cat\ observed} - k_{cat\ H_2O}} = \frac{1}{k_{cat\ Tris} - k_{cat\ H_2O}} \left(1 + \frac{K_T}{Tris}\right)$$

where $k_{cat\ H_2O}$ is the off-rate of Pi, $k_{cat\ Tris}$ is the off-rate of Tris phosphate, and K_T is the Michaelis constant for Tris binding to the E-P form. The predicted linear plots were seen with data obtained at both 10° C and 25° C. At 10° C, $k_{cat\ Tris} = 27\ sec^{-1}$, $k_{cat\ H_2O} = 3\ sec^{-1}$, and $K_T = 0.18$ M. At 25° C, $k_{cat\ Tris} = 100\ sec^{-1}$, $k_{cat\ H_2O} = 10\ sec^{-1}$, and $K_T = 0.50$ M. The results suggest that the increased k_{cat} as a function of Tris concentration is due to the increased off-rate of the product Tris phosphate formed in the presence of Tris. Although the Tris base is supposed to facilitate the hydrolysis of E-P via nucleophilic attack, the increased rate of hydrolysis is not responsible for the increase in k_{cat}. Support for this argument is derived from transient kinetic studies in which a single transient rate constant is obtained in the "burst" kinetics, which indicates that the product release step is much slower than that of the dephosphorylation step.

(2) Several lines of evidence from various laboratories suggest that the asparaginase-catalyzed reaction proceeds via an aspartyl-enzyme intermediate. The proposed covalent intermediate, however, has never been isolated. Our attempts to trap or isolate the intermediate at neutral pH and room temperature by a number of methods such as $NaBH_4$ reduction, trichloroacetic acid (TCA) precipitation, and gel electrophoresis of enzyme samples incubated with labeled asparagine or aspartate failed to produce positive results. When the reaction conditions were changed to lower pH and temperature to minimize the rate of hydrolysis, the aspartyl-enzyme intermediate was implicated in several experimental observations: (i) when asparaginase was incubated with [^{14}C]-asparagine at pH 5.5, 4° C, for one hour, passed through a Sephadex G-25 column, and then precipitated by TCA, the washed precipitate contained high radioactivity (30,000 cpm per mg protein). The precipitate was washed with TCA up to ten times without any noticeable loss of counts; (ii) when time course of the reaction was followed, the TCA precipitable counts gradually reached a steady phase, then declined and attained a new steady phase. This indicates that the TCA precipitable counts were not non-covalently bound [^{14}C]-Asp somehow trapped by the protein precipitate. Rather, it reflected a true intermediate that reached a steady-state when Asn was in excess, and then declined as substrate was depleting. The final steady-state level is consistent with the notion that the aspartyl-enzyme intermediate can also be formed from the enzyme and the product aspartate; (iii) incubation of [^{14}C]-Asp with asparaginase also yielded TCA precipitable counts that could not be washed away; (iv) when the labeled protein was digested with pepsin and then passed through Sephadex G-25 column, a major labeled peptide fraction was identified. Work is in progress to characterize the peptide and to determine the amino acid residue to which the ester linkage with the aspartyl

group is formed. The proposed overall reaction scheme is as follows:



(3) The kinetic mechanism of asparagine synthetase is of interest for several reasons: (i) the presence of this enzyme in certain leukemic cells is responsible for the cells' resistance to treatment with asparaginase. An understanding of the kinetic mechanism may shed some light on the type of inhibitor that may be used to suppress the asparagine synthetase activity; (ii) like several other glutamine amidotransferases, this enzyme exhibits glutaminase activity. The mechanism of action of this class of enzymes has not been adequately studied. Whether the glutaminase reaction occurs on a separate subunit in a "two-site" ping-pong reaction has not been investigated.

Detailed analysis based on product inhibition patterns show that the kinetic patterns are most consistent with a Uni Uni Bi Ter Ping-Ping-Theorell-Chance mechanism with the formation of E-NH_3-Asn and E-NH_3-ASP-AMP complexes. The "two-site" ping-pong mechanism does not agree with the observed inhibition patterns and is therefore ruled out.

(4) A set of formulas for the rapid computation of the exact number of King-Altman patterns for the derivation of steady-state rate equations has been previously reported. The formulas were presented without precise mathematical proof, though their validity could be verified by comparing the results with those obtained by conventional methods. Mathematical proofs can now be provided by considerations of the theory of combinations. An example of such a proof for the simplest case is shown below.

For a geometric diagram (reaction scheme) consisting of two subfigures, A and B (Figure 1), the exact number of noncyclic King-Altman patterns, P, is given by

$$P = ab - \ell_{AB}^2$$

where a and b are the number of lines in A and B, and ℓ_{AB} is the number of lines in the common boundary between A and B. The noncyclic King-Altman patterns can be considered as having two types: those containing all the lines in the common boundary and those containing $(\ell_{AB} - 1)$ lines. Any valid pattern must include at least $(\ell_{AB} - 1)$ lines from the common boundary because the pattern must flow through all the corners (enzyme species) of the geometric figure.

Figure 1

When the complete boundary is present, the number of noncylic patterns is given by

$$C^{\,a-\ell_{AB}}_{\,a-\ell_{AB}-1} \cdot C^{\,b-\ell_{AB}}_{\,b-\ell_{AB}-1} = (a-\ell_{AB})\,(b-\ell_{AB})$$

$(a - \ell_{AB})$ is shown as dashed lines in subfigure A and $(b - \ell_{AB})$ as dashed lines in subfigure B (Figure 2).

Figure 2

When the patterns contain $(\ell_{AB} - 1)$ lines from the common boundary, the number of noncyclic patterns is given by

$$C^{\,a-\ell_{AB}+b-\ell_{AB}}_{\,a-\ell_{AB}+b-\ell_{AB}-1} \cdot C^{\,\ell_{AB}}_{\,\ell_{AB}-1} = (a+b-2\ell_{AB})\,\ell_{AB}$$

Figure 3

$(a + b - 2\ell_{AB})$ is equivalent to the number of lines in the perimeter (shown as solid lines in Figure 3). Thus, the total number of valid King-Altman patterns is

$$P = (a - \ell_{AB})\,(b - \ell_{AB}) + (a + b - 2\ell_{AB})\,\ell_{AB}$$
$$= ab - \ell_{AB}^2$$

Mathematical proofs for all other formulas can be demonstrated by the same approach. Proof for a simplifying "conversion" procedure for certain symmetrical geometric figures previously reported can be obtained by simple substitution of appropriate equations.

The formulas described above are applicable to the calculation of the total number of reversible King-Altman patterns. However, enzyme kinetic reaction schemes often contain irreversible steps because in most cases only the initial velocity of the reaction in one direction is of interest. In the presence of irreversible steps, the number of King-Altman patterns will vary for individual enzyme species. It is desirable, therefore, to calculate the individual number of patterns so that the exact number of terms to be expected for each determinant can be verified. A method has been developed for such calculations, based on principles analogous to the graph-theory approach used by Volkenstein and Goldstein (Biochim. Biophys. Acta 115, 471, 1966).

As an example, consider the reaction scheme shown in Figure 4. Because

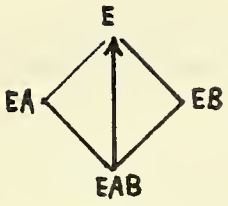


Figure 4

of the presence of the irreversible EAB → E step, only E contains all the valid King-Altman patterns. This is because all the branches lead towards E. The EAB → E branch does not contribute to the King-Altman patterns of EAB, since it leads away from EAB. Thus, for EAB, only the perimeter shown in Figure 4 need be considered in calculating the noncyclic patterns:

$$P = (a + b - 2\ell_{AB}) = 4$$

For EA, there are two types of noncyclic patterns: those containing the irreversible branch, and those not containing it (the number is the same as that obtained for EAB, 4). To compute the number of patterns containing the irreversible branch, all the possible flow patterns (without forming a loop) connecting the irreversible branch to th enzyme species under consideration - in this case, EA → must be drawn. For EA, only one such flow pattern exists (Figure 5). Enzyme species not involved in the flow pattern (EB) retain branches going away from them (Figure 5, dashed lines). The pathway EAB → E → EA is then compressed into a single point (Figure 6), and the number of King-Altman patterns is computed from the compressed figure using the appropriate formula previously described. Here the compressed figure has two lines, and

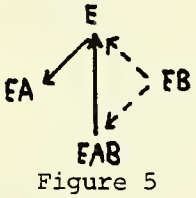


Figure 5

$$P = 2$$

The total valid patterns for EA are simply

$$P = 4 + 2 = 6$$

Figure 6

For more complicated cases where more than one irreversible step is present, one must take into account all the possible flow patterns that can be constructed from one irreversible step at a time and from various combinations of several and all irreversible steps. It should be noted that all the other existing methods do not permit the calculations of patterns for individual enzyme species and therefore have limited usage.

Proposed Course of Research

(1) Purification of uridylyltransferase and uridylyl-removing enzyme in the *E. coli* glutamine synthetase system.

(2) Interaction of calmodulin and its binding protein with the cyclic nucleotide phosphodiesterase, Ca^{++}-dependent protein kinases, and the microtubule systems.

Publications

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Equations. In Methods in Enzymology, Vol. 63A. New York, Academic Press, 1979, pp. 54-84.

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Rhee, S. G., Huang, C. Y., Chock, P. B., and Stadtman, E. R.: New Methods for the Colorimetric Assay of P_{IID} Regulatory Protein, Uridylyltransferase, and Uridylyl-Removing Enzyme in Glutamine Synthetase Cascade, Anal. Biochem. 90, 752-766, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00225-02 LB

PERIOD COVERED
October 1, 1978 to September 30, 1979

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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Other:	Earl R. Stadtman	Chief, Laboratory of Biochemistry	LB	NHLBI

COOPERATING UNITS (if any)

None

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Extracts of Klebsiella aerogenes inactivate glutamine synthetase. The reaction has characteristics similar to the cytochrome P-450-dependent mixed function oxidations. Ascorbic acid has been used in model systems of these oxidations. Ascorbic acid rapidly inactivates glutamine synthetase with characteristics of the Klebsiella extract: (1) It is oxygen-dependent; (2) Catalase blocks the reaction, implying participation of hydrogen peroxide; (3) Ferric salts stimulate inactivation while chelating agents prevent inactivation.

Of the four amino acids which are feedback inhibitors of glutamine synthetase, only histidine affected the ascorbate-mediated inactivation. Histidine could completely protect the enzyme. A Kd for histidine was estimated from the protective effect (0.26 mM).

The substrates glutamate and ATP had differential effects on the two forms of the enzyme. Unadenylylated enzyme was protected from inactivation, while the adenylylated enzyme became more susceptible to inactivation.

Project Description

Objectives: Cell-free extracts from Klebsiella aerogenes inactivate glutamine synthetase through an oxygen-dependent reaction. The primary objectives of this project are determination of the chemical change which occurs on inactivation of the glutamine synthetase; purification and characterization of the inactivating component(s) of the extract; and assessment of the physiological significance and control of the inactivation.

Major Findings

1. Rationale

Most of this year's effort was directed towards development of chemical models of the Klebsiella inactivating system. This became feasible when it was found that defined redox systems could mimic the extract-mediated inactivation.

The Klebsiella extract has characteristics similar to the P-450 mixed function oxidases which function (in part) as drug-metabolizing systems in mammals: inactivation of glutamine synthetase by the extract required NAD(P)H and oxygen. Ferric salts stimulated the inactivation. Chelators such as EDTA and o-phenathroline prevented inactivation. Catalase or horseradish peroxidase also prevented inactivation, implying the participation of hydrogen peroxide.

Ascorbic acid has long been known to mimic many aspects of the microsomal drug-metabolizing system. Studies with ascorbate-containing model systems have helped elucidate the more complex enzymatic systems. For this reason, ascorbate-induced inactivation was studied as a potential model of the Klebsiella system.

Although only the ascorbate results will be considered in detail here, other chemically defined redox systems were also found to inactivate glutamine synthetase. These included: (1) NADH and menadione; (2) NADH and sodium nitroprusside; (3) Ferric-EDTA and hydrogen peroxide. The inactivations required oxygen, were stimulated by iron salts, and were inhibited by chelating agents and catalase. (Catalase was inhibited by nitroprusside and did not affect that system.)

2. Ascorbate Rapidly Inactivates Glutamine Synthetase

Both adenylylated and unadenylylated glutamine synthetase, purified from Escherichia coli, were inactivated. The rate and extent of inactivation depended on the concentration of ascorbate. At the optimal pH of 7.2, 50% of glutamine synthetase activity was lost in 15 minutes. This loss was independent of the concentration of glutamine synthetase over the 50-fold range tested (24-1205 nM). During the reaction, less than 5% of the ascorbate was consumed.

Auto-oxidation of ascorbate produces hydrogen peroxide and dehydroascor-

bate. Neither compound alone or together caused any loss of glutamine synthetase activity.

3. Effect of Oxygen-Metabolizing Enzymes and Radical Scavengers

As in the Klebsiella system, catalase or horseradish peroxidase completely protected glutamine synthetase from ascorbate-mediated inactivation. Also, analogous to the Klebsiella system, superoxide dismutase had no effect. These results imply a role for hydrogen peroxide and eliminate superoxide anion as the inactivating species. Since superoxide dismutase converts superoxide to hydrogen peroxide, it was still possible that superoxide was an intermediate in the reaction. Xanthine oxidase was used to generate superoxide anion, in the absence of ascorbate. Glutamine synthetase was entirely stable in this milieu, with or without added superoxide dismutase.

Radical scavengers were tested in the ascorbate system. Ethanol, 600 mM, and BHT, 1 mM, had no effect. Mannitol, 250 mM, actually stimulated loss of glutamine synthetase by 50%. Although these results do not support a free radical mechanism, neither do they a rule out a "site-specific" free radical generator which may be inaccessible to the scavengers.

4. Effect of Metals and Chelators

Here, the ascorbate system precisely mimics the Klebsiella system: ferric salts stimulated inactivation, with 0.2 mM more than doubling the rate. Manganese 0.4 mM, inhibited the reaction by 80%. o-Phenanthroline completely protected glutamine synthetase at 0.1 mM. Inactivation was restored by addition of an excess of ferric chloride.

EDTA, 1 mM, also protected glutamine synthetase from ascorbate-mediated inactivation. Excess ferric chloride did not restore inactivation. (This curious result was previously noted in the Klebsiella system.) However, excess cupric chloride did restore inactivation. Cupric chloride without EDTA did not stimulate inactivation.

These effects occurred with both unadenylylated ($\bar{n}$ = 1) and adenylylated ($\bar{n}$ = 10) enzymes.

5. Native and SDS Slab-Gel Electrophoresis

Native and SDS gel electrophoresis was performed on glutamine synthetase treated with varying concentrations of ascorbate, from 0 to 100 mM. Loss of glutamine synthetase activity ranged from 0 to 96%.

On the SDS gel, virtually all protein in each sample ran at 50,000 MW, indicating that proteolysis was not involved in inactivation. (A faint band of MW less than 50,000 did appear in the ascorbate-treated samples.)

On the native gel, almost all protein in each sample ran at 600,000 MW, indicating that dissociation of the native dodecamer did not occur during

SF

inactivation. (Faint, multiple bands of lower MW did appear in the ascorbate-treated samples.)

6. Effect of Amino Acids

Cumulative feedback inhibition has been studied in detail for glutamine synthetase. Four amino acids are feedback inhibitors and participate in cumulative feedback inhibition (alanine, glycine, histidine, and tryptophan).

The twenty common amino acids were tested for their effect on the inactivation of glutamine synthetase ($\bar{n}$ = 1) by 15 mM ascorbate. The l-isomers of the amino acids were tested at 5 mM.

Reproducible effects were seen with several amino acids, but only 4 affected the inactivation by more than 10%. Isoleucine stimulated inactivation by 11%, while glutamate stimulated inactivation by 60%.

Cysteine inhibited inactivation by 69%. Histidine inhibited inactivation by 72%. The effect of histidine was investigated in more detail, since there is some question whether it actually binds to glutamine synthetase as a feedback inhibitor.

The inhibition of ascorbate-mediated inactivation was dependent on the concentration of histidine. The concentration dependence was similar to that for feedback inhibition of the enzyme observed by Woolfolk and Stadtman (Arch. Biochem. Biophys. 118:736, 1976). Histidine could completely protect the glutamine synthetase from inactivation by ascorbate, permitting a more precise estimate of its presumed binding to the enzyme. Assuming that histidine binds to the enzyme, and that binding prevents inactivation by ascorbate, the Kd for histidine is 0.25 mM. However, since the d- and l-isomers of histidine had equal effects, histidine may not act by direct binding to the enzyme.

7. Effect of Substrates and Products

Substrates and products were tested for their effect on the inactivation of unadenylylated ($\bar{n}$ = 1) and adenylylated ($\bar{n}$ = 12) enzyme by 15 mM ascorbate.

Of the products, 1 mM glutamine or phosphate had little effect on the extent of inactivation. ADP markedly increased the loss of activity. With no ADP, the loss was 50%; with 1 mM Mg-ADP, this increased to 90%. There was no major difference in the effect of the products on GS_1 compared to GS_{12}.

For the substrates, 50 mM ammonium chloride had little effect, alone or with the other substrates. MgATP, 1 mM, stimulated inactivation, as did 30 mM glutamate. The effect was more marked on the adenylylated enzyme than on the unadenylylated form.

The most intriguing finding was a differential effect on GS_1 and GS_{12} in the presence of both MgATP and glutamate. GS_1 was protected while the inactivation of GS_{12} was stimulated:

Additions	% of Activity Lost	
	GS_1	GS_{12}
None	50	46
NH_4Cl, 50 mM	53	56
Mg-ATP, 1 mM	60	76
Glutamate-Na, 30 mM	64	73
NH_4Cl and MgATP	64	78
NH_4CL and Glutamate	66	76
MgATP and Glutamate	15	77

So, in the presence of its substrates, the enzymatically active form (unadenylylated) of glutamine synthetase is protected from attack by ascorbate, while the inactive form (adenylylated) becomes more susceptible. The ascorbate system could be a model for a "physiologically sensible" inactivation mechanism, controlled in part through the exquisitely-regulated adenylylation.

8. Ferrous Iron Inactivates Glutamine Synthetase

The characteristics described above suggested that the ascorbate reaction was a redox system involving iron. Ferric salts stimulated inactivation by ascorbate, but had no effect in the absence of ascorbate.

Ferrous salts were studied because ascorbate is known to be able to reduce ferric iron to the ferrous state. Ferrous sulfate rapidly inactivated glutamine synthetase in the absence of ascorbate. At 1 mM concentration, 80% of the activity was lost when the reaction stopped at 10 minutes. Even 1 µM ferrous sulfate led to a loss of 10% of the initial activity. (The enzyme subunit concentration was about 6 µM.)

Catalase had no effect on the inactivation, but exclusion of oxygen prevented inactivation. o-Phenanthroline and EDTA also blocked inactivation, and excess ferrous sulfate restored inactivation in both cases. The radical scavengers, ethanol and BHT, had no effect, while mannitol stimulated inactivation. These effects paralleled those seen with the ascorbate inactivation.

Concentrations of hydrogen peroxide less than that of the ferrous sulfate led to an increased loss of glutamine synthetase activity. Excess concentrations of peroxide actually protected the enzyme.

These results suggest that the ascorbate-mediated inactivation could involve the reduction and oxidation of iron. Ferrous ion might bind to the divalent cation site(s) on glutamine synthetase. Oxidation to the ferric state could generate a free radical which reacts with the protein. This would be a "site-directed" free radical mechanism.

Significance to Biomedical Research

In previous reports, we noted that extracts of Klebsiella inactivate glutamine synthetase. With prolonged incubation degradation of the glutamine synthetase occurs, as indicated by the loss of cross-reacting material. Enzyme inactivation could be an initial step in the degradative pathway.

The net concentration of any protein represents the balance point of its synthesis and degradation. The biochemical mechanisms of protein synthesis are known in detail, but very little is known of the pathways of protein degradation.

Knowledge of the mechanism of degradation of specific proteins could be especially important. Abnormal protein degradation occurs in several disease states -- muscular dystrophy is probably the best known. The degradation of specific proteins is particularly important during growth and development of the fetus and of children.

In general, physicians have been able to consider and manipulate the synthesis of proteins, but they rarely consider degradation. For example, there are severe problems of nutrition in premature infants and in patients with cancer. Clinical treatment in recent years has emphasized increased intravenous input of carbohydrate, amino acids, and lipids, in an attempt to favorably influence the synthesis of tissue and especially of protein. An understanding of protein degradation might permit a decrease in the degradative loss of tissue.

Proposed Course of Research

The chemical change(s) in glutamine synthetase caused by ascorbate will be studied. Coupled with knowledge of the system outlined above, this information should aid in purification of the inactivating system from Klebsiella.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00226-02 LB

PERIOD COVERED
October 1, 1978 - September 30, 1979

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 and Bioenergetics	LB NHLBI

COOPERATING UNITS (if any)

None

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1.4	1.1	0.3

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Selenocysteine has been demonstrated in the selenium-dependant formate dehydrogenase of Methanococcus vannielii. Evidence has been gathered that suggests that nicotinic acid hydroxylase from Clostridia barkerii is a selenium protein.

Project Description

Objectives: To elucidate the role of selenium in selenoproteins and to determine the biosynthetic pathway of their synthesis. This entails studying the form of selenium in these proteins and the location of the selenium-containing residues within the protein.

Major Findings: (1) The large membrane-bound complex form of formate dehydrogenase from Methanococcus vannielii is known to contain selenium. The selenium-containing component was shown to be selenocysteine by characterizing two alkylated derivatives after enzymatic and chemical hydrolysis. Se-Carboxymethylselenocysteine and Se-Carboxamidomethylselenocysteine were characterized by co-chromatography on an amino acid analyzer and co-migration in two solvents during thin layer chromatography with authentic compounds.

(2) The enzyme, nicotinic acid hydroxylase from Clostridium barkerii has been shown to be a selenium-containing protein by the following criteria: co-elution of selenium with activity after preparative polyacrylamide gel electrophoresis, sucrose density centrifugation and gel filtration. The selenium-containing component has not yet been characterized.

(3) Commercial C^{14} and H^{3} labeled selenocysteine is not readily available so a project was undertaken to synthesize these compounds. The most satisfactory procedure involved the acetylation of labeled serine to form O-acetylserine and then incubation with E. coli O-acetylserine sulfhydrolase and excess sodium selenide which resulted in the almost quantitative conversion of serine to selenocysteine.

(4) Attempts were made to feel $^{3}H + ^{14}C$ selenocysteine to Clostridium sticklandii in order to follow the incorporation of the selenocysteine into the seleno-component of glycine reductase. While it was possible to show the incorporation of the selenium, there was no discernible incorporation of the labeled backbone. C. sticklandii is an amino acid fermenting organism that grows in media rich in amino acids, thus it is possible that there was extensive metabolism of the selenocysteine prior to incorporation. Similar experiments are planned using other organisms which do not actively ferment amino acids but do contain the selenoproteins, formate dehydrogenase or nicotinic acid hydroxylase, providing the selenium occurs as selenocysteine in this latter case.

(5) The location of the selenocysteine moiety within glycine reductase selenoprotein A, particularly in relation to the two cysteinyl residues, may be very useful in determining the role of selenium in this protein. Preliminary experiments with small amounts of protein indicated that it might be possible to find a single polypeptide containing both the two cysteinyl and selenocysteinyl residue after chymotrypsin digestion. Attempts to isolate a significant amount of this peotide were unsuccessful as the amount of peptide formed during chymotripsin treatment dropped as the digestion was scaled up. Endoproteases with greater specificity will be screened for their ability to produce a selenopeptide which contains one or two of the cysteinyl residues.

Abstract: Bacterial Selenoproteins. T.C. Stadtman, G.L. Dilworth and C.S. Chen. 3rd Int. Symp. on Selenium and Tellurium Chemistry.

Publications: J.B. Jones, G.L. Dilworth and T.C. Stadtman. Identification of Selenocysteine in the Selenium-dependant Formate Dehydrogenase of Methanococcus vannielii. Arch. Biochem. Biophys., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00227-02 LB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Interaction of the Dye Cibacron Blue F_3GA with Glutamine Synthetase

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

P.I.:	Mary Marcia Federici	NIH Postdoctoral Fellow	LB	NHLBI
Other:	E. R. Stadtman	Chief, Laboratory of Biochemistry	LB	NHLBI

COOPERATING UNITS (if any)

None

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

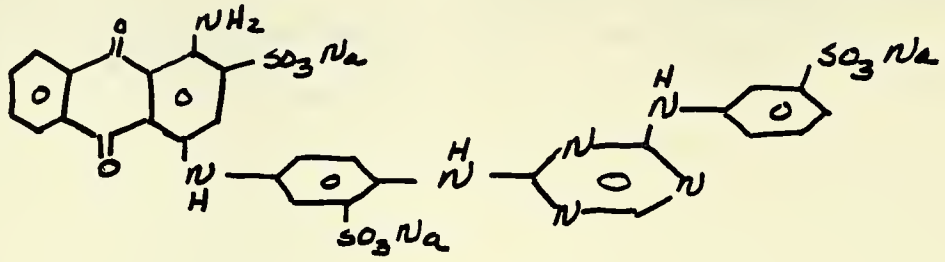
SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this investigation is to study the effects of the protein glutamine synthetase on the visible spectrum of the dye Cibacron Blue F_3GA. A dye difference spectrum with the native (taut) form of glutamine synthetase is characterized by a maximum at 640 nm and a minimum at 700 nm. The spectra obtained when the dye binds to the relaxed and subunit forms of glutamine synthetase (two known structural forms of the protein) differ from each other and clearly differ from the characteristic spectrum obtained with the native (taut) GS. Purification of the dye resulted in the separation of three chromatographically distinct subfractions of Cibacron Blue. The difference spectra obtained when each of these subfractions binds to taut, relaxed, or subunit forms of GS clearly differ from each other. These results indicate that dye difference spectroscopy can be used to monitor changes in protein conformation associated with the relaxation and subunit dissociation reactions.

PHS-c040
(Rev. 10-76)

Project Description and Background

Previous studies by others have shown that the dye, Cibacron Blue F_3GA, (structure shown below) can be used for probing the nucleotide binding sites in certain proteins.



The measurement of dye versus dye plus enzyme spectrum for various kinases and dehydrogenases yields a characteristic difference spectrum with a positive maximum at 660-680 nm. Addition of nucleotides to the dye plus protein sample displaces the dye from the nucleotide binding site and results in the loss of the observed difference spectrum.

Escherichia coli glutamine synthetase is a multi subunit enzyme which requires the nucleotide, ATP, in its catalytic formation of glutamine from glutamic acid and NH_3. Several structural forms of the enzyme are known to exist. Removal of divalent cations from the native (taut) form of GS converts the enzyme to a relaxed (catalytically inactive) configuration that is characterized by exposure of sulfhydryl groups and a shift of aromatic amino acid residues from a hydrophobic to a hydrophilic environment. Also, at pH (> 8.5) or in the presence of 2 M urea, the relaxed enzyme is dissociated to subunits of 50,000 MW. It is the scope of this investigation to explore the interaction of Cibacron Blue F_3GA with these known structural forms of glutamine synthetase.

Major Findings

Spectral Characteristics of the Interaction of Cibacron Blue with GS

(A) Studies with Unfractionated Dye

A distinct difference spectrum is characteristic of the binding of the dye to the native (taut) form of glutamine synthetase which is characterized by a maximum at 640 nm and a minimum at 700 nm. Relaxation of the enzyme in the presence of dye, by the addition of EDTA, results in an alteration in the amplitude at the maximum and minimum of the difference spectrum as compared to the one obtained with the native (taut) form of the protein. This is supported by the difference spectrum obtained when the relaxed enzyme plus dye

is compared to taut enzyme plus dye. Addition of divalent metal cation to the relaxed enzyme in the presence of dye results in the regeneration of the original (taut enzyme) spectrum. Good agreement with the above results is obtained if the relaxation and tightening reactions are carried out in the absence of dye and then the difference spectrum is obtained.

At high pH (> 8.5) the relaxed enzyme is dissociated to individual subunits of 50,000 MW. Treatment of the relaxed enzyme in the presence of dye with a mixture of Tris/KOH (pH 8.5-9.5) results in significant alterations of the difference spectrum as compared to that obtained with either the relaxed or native (taut) protein. The difference spectrum for the dissociated protein is characterized by a time-dependent shift in the maximum from 640 to 650-660 nm accompanied by an increase in amplitude. Another characteristic is a dramatic decrease in the amplitude at the minimum of 700 nm.

Control experiments were performed and demonstrated that addition of $MnCl_2$ or EDTA had no effect on the spectrum of the dye alone in the standard system: 40 mM Hepes pH 7.0 or 7.5, 100 mM KCl. (Omission of KCl from the standard buffer system results in large changes in the spectrum of the dye after the addition of either $MnCl_2$ or EDTA.)

(B) Purification of Cibacron Blue F_3GA and Separation of the Major Subfractions of the Dye

Thin layer chromatography of Cibacron Blue F_3GA (obtained from Ciba Geigy) on precoated silica plates in the solvent, t-butanol / methyl ethyl ketone / H_2O / NH_4OH 40 : 30 : 20 : 10, demonstrated that at least six different components of the dye are present in commercial dye preparations. Using a preparative silica gel TLC system, the major dye component was isolated and purified. Rechromatography of this major band in tetrahydrofuran / H_2O 48 : 7 demonstrated two components which migrated very closely together in this system at approximately equal concentration. TLC analysis of the unfractionated dye in T H F / H_2O 48 : 7 demonstrated eight distinct subfractions of the dye (3 major subspecies).

A preparative procedure was devised for purifying larger quantities of the dye utilizing preparative silica gel columns. The dye was routinely acidified with $NAHSO_4$ and then applied to a silica column equilibrated with ethyl acetate / 1-butanol 4 : 1. Elution was initially carried out with a solvent system of the same composition and then the solvent composition was switched to ethyl acetate / 1-butanol 2.7 : 1 for the remainder of the experiment. Utilizing the above procedure the three major subfractions of the dye were resolved and purified to chromatographically homogeneous material.

Proposed Course of Action

The three major subfractions of the dye will be analyzed structurally and with respect to binding to the enzyme. Representative studies planned for the characterization of these subfractions include:

1. Assessment of the binding of the dye fractions to native (taut) form of GS with respect to stoichiometry and binding constants.

2. Evaluation of each of the three fractions as monitors of the relaxation and dissociation reactions.

3. ADP reversibility of binding of the dye fractions to the enzyme.

4. Series of studies on the kinetics of relaxation and dissociation reactions since spectral changes thus far monitored are a time and temperature dependent phenomenon.

5. Influence of the state of adenylylation and various effectors on dye binding.

Collaborative Work

In addition, I have collaborated with Dr. Emilio Garcia and Dr. Sue Goo Rhee on a project involving gene cloning for the enrichment of one of the proteins involved in the covalent modification of glutamine synthetase in E. coli. ColE1 hybrid plasmids were used to obtain increased levels of two of the components involved in the covalent modification of glutamine synthetase and thus complement the defect in glnD mutants. In two of these hybrid plasmid-containing strains, the increase in UTase levels was correlated with a parallel increase in uridylyl-removing enzyme levels. In addition, two plasmids that complement strains with mutations in the glnA gene, the structural gene for glutamine synthetase, are also capable of complementing the defect in glnD mutants.

Publications

Garcia, E., Federici, M., Rhee, S. G., and Berberich, M. A.: Gene Cloning for Enrichment of an Enzyme of the Covalent Modification Cascade of Glutamine Synthetase in E. coli. J. Bacteriol., submitted for publication.

Federici, M. M., and Stadtman, E. R.: Interaction of Cibacron Blue F_{3GA} with Glutamine Synthetase: Use of the Dye to Monitor Structural Alterations in the Protein. Abst. XIth International Congress of Biochemistry, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00228-02 LB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Enrichment of Enzymes of the Covalent Modification Cascade of Glutamine Synthetase by Escherichia coli Carrying Hybrid ColE1 Plasmids

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:	E. R. Stadtman	Chief, Laboratory of Biochemistry	LB	NHLBI

COOPERATING UNITS (if any)
None

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

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

To facilitate construction of strains producing elevated amounts of the proteins involved in covalent modification of glutamine synthetase (GS), we have characterized mutants of Escherichia coli which require glutamine for growth. Using a class of mutants (glnD) that lack uridylyltransferase (UTase) activity, we have screened a collection of 2,000 E. coli strains carrying hybrid ColE1 plasmids (L. Clarke and J. Carbon (1976) Cell 9, 91-99) for those capable of correcting the glutamine requirement of the glnD strains. Two hybrid plasmid-containing strains capable of complementing the glnD mutations were found to overproduce UTase by 14- to 25-fold. In both of these strains the increase in UTase was paralleled by a concomitant increase in the levels of uridylyl-removing (UR) enzyme. Strain JA200/pLC 38-39, which produces 25-fold the normal level of UR-UTase, will be used for the isolation of this (these) enzyme(s).

PHS-604C
(Rev. 10-76)

Project Description

Objectives

The immediate objective of this project is to obtain strains of Escherichia coli which are enriched in the intracellular concentrations of the proteins involved in the covalent modification of GS. The use of cloning (extrachromosomal propagation of selected segments of DNA in bacterial cells) allows the isolation and purification of specific genes and gene products. We shall make use of a strain in which the UTase-UR gene(s) is(are) carried in a ColE1 hybrid plasmid to attempt the purification of this enzyme. Together with a detailed biochemical characterization, such purification will provide a definite answer as to whether the UTase and UR activities reside on a single enzyme (polypeptide).

Major Findings

A. Characterization of glnD (UTase)$^-$ Mutants of Escherichia coli K12

Two ICR-induced glutamine-requiring mutants previously isolated by Dr. Maryanne Berberich (this laboratory) have been characterized. These glnD mutants have levels of GS activity which are one-third to one-half those found the wild type strain and accumulate GS which is highly adenylylated under all growth conditions. Assays in dialyzed extracts prepared from these strains showed undetectable levels of UTase activity, suggesting that the presence of highly adenylylated GS is the consequence of the lack of UTase activity in these mutants. Mapping of the mutations leading to glutamine requirement in these mutants indicated that the lesion is located at 4 minutes in the E. coli chromosome, and 90% linked to the dapD locus by P1-mediated transduction. In addition, these strains which show slow or reduced capability of growth in the absence of glutamine are severely inhibited by 10 to 20 mM glutamate. Thus, these mutants are analogous to glnD mutants previously described in Salmonella (J. Bacteriol. 134, 1046-1055, 1978) and E. coli (J. Bacteriol. 134, 569-577, 1978).

B. Identification of Hybrid ColE1 Plasmids Carrying the glnD$^+$ Gene

Using direct mating on plates, it was found that two hybrid ColE1 plasmids out of 2,000 in the collection restored the growth of glnD mutants in minimal medium containing 15 mM glutamate. These two hybrid plasmids also complemented mutants defective in a closely linked, but unrelated gene, dapD, establishing that complementation by these plasmids occurs by providing the mutants with a short wild type chromosomal segment corresponding to the 4 minute region of the E. coli chromosome.

C. Levels of UTase and UR Enzyme

Because hybrid ColE1 plasmids can be maintained at 10 to 20 copies per chromosome, the presence of a given gene on the plasmid can lead to the over-

production of the gene product encoded by the hybrid plasmid DNA by a gene dose effect. Examination of the levels of UTase in the two strains carrying the glnD$^+$ gene in the hybrid plasmid showed that these strains have 14 to 25-fold higher levels of UTase than those in the nonplasmid-containing strains. Appropriate mixing experiments with dialyzed streptomycin supernatant from plasmid-containing and nonplasmid-containing strains indicated that the increased UTase activity observed in the ColE1 glnD$^+$ plasmid-bearing strains is not due to the presence of an activator in these extracts. Mixing gave the simple additive results expected from mixing in which no activator or inhibitor of UTase activities are present. Analogous experiments in which the levels of UR activity were measured in these ColE1 glnD$^+$ strains indicated that UR activity increases in parallel with UTase activity. These findings are consistent with previous biochemical and genetic evidence which suggested that these two activities may reside on the same protein (i.e., may be encoded by the same gene).

The two ColE1 glnD$^+$ hybrid plasmids have been isolated and their molecular weight determined. Hybrid plasmid pLC 6-32 had a molecular weight of 15 megadaltons, and pLC 38-39 had a molecular weight of 10 megadaltons. The smaller pLC 38-39 was associated with higher enzyme expression. However, it is not known if this is due to a higher copy number for this plasmid. From the molecular weight determinations, it can be inferred that the size of the inserted hybrid DNA carried by these plasmids is 5.8 megadaltons for pLC 38-39, and 10.8 megadaltons for pLC 6-32, corresponding to about 8,000 to 16,000 base pairs, respectively. This represents enough genetic material to encode 10-20 polypeptides of 20,000 molecular weight, respectively.

D. GS Actvity and State of Adenylylation in Cells Carrying ColE1 glnD$^+$ and ColE1 glnA Hybrid Plasmids

Since ultimately the action of UTase is expressed in the level of GS adenylylation, the activity and state of adenylylation in the strains carrying hybrid plasmids were determined. The glnD mutants have GS levels which are one-third to one-half those of the wild type and accumulate highly adenylylated GS under all growth conditions. When these mutants carry a ColE1 glnD$^+$ hybrid plasmid, both the normal modulation of the state of adenylylation of GS and the wild type levels of GS are restored. This restoration of deadenylylating activity is consistent with the recovery of UTase caused by the presence of a functional glnD$^+$ gene in the hybrid plasmid, and it explains the ability of this hybrid plasmid to complement the glnD mutations.

In the course of screening the collection of ColE1 hybrid plasmids, it was found that plasmids that carry the glnA$^+$ gene (the structural gene for GS) also were capable of complementing the glnD mutation. However, rather than causing a change in the state of adenylylation of the GS in the glnD mutants, the presence of this ColE1 glnA$^+$ hybrid plasmid caused an increase in the levels of GS over and above the levels found in the wild type strain, but the enzyme remained highly adneylylated. Thus, it appears that the ability to complement the glnD mutants by these ColE1 glnA$^+$ hybrid plasmids is related

to their ability to increase the absolute levels of GS in the mutant, rather than by changing the state of adenylylation.

In contrast with the gene dose effect obtained in ColE1 *glnD*$^+$ strain with respect to the level of UTase, strains which carry multiple copies of *glnA*$^+$ (the structural gene for GS) cannot significantly increase the level of GS beyond those achieved during derepression of GS by the haploid strain. This is consistent with previous observations that the synthesis of GS requires the *glnF* gene product (Proc. Natl. Acad. Sci. 74, 1662-1666, 1977) and therefore that its levels cannot increase simply as a function of gene dose.

Proposed Course of Action

In collaboration with Dr. Sue Goo Rhee, we intend to make use of the newly isolated strain carrying multiple copies of the UTase-UR gene(s), which has levels of this (these) enzyme(s) 25-fold greater than those in the haploid strain, to purify these two activities. Using a strain which has been constructed by incorporating the ColE1 *glnD*$^+$ hybrid plasmid into a minicell-producing strain, it will be possible to selectively label (with ^{35}S) UTase. This labeled UTase may be used to facilitate the purification of UR-UTase.

Work will be initiated in trying to construct a strain carrying multiple copies of the gene encoding ATase which presumably may enrich the intracellular levels of this enzyme. Feasibility of this project will be dependent on:

(1) Adaptation of a method for whole cell assay determination of the state of adenylylation of GS to minute amounts of cell grown in Micro Test II culture plates.

(2) The use of mutants defective in ATase currently are being characterized. In collaboration with Dr. Sue Goo Rhee, we are presently carrying out partial purification of components of adenylylation system which are required for assaying UTase and UR during their purification.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00229-01 LB

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (30 characters or less)

Control of Adenylylation of Glutamine Synthetase in Permeabilized E. Coli Cells

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

P.I.:	Umberto Mura	Visiting Fellow	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:	PROFESSIONAL:	OTHER:
0.9	0.8	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 emphasis of this project is the study of the adenylylation and deadenylylation of glutamine synthetase within bacterial cells. Although this project has just begun, preliminary results indicate E. coli cells can be effectively permeabilized by the non-ionic detergent, Lubrol. These treated cells are readily permeable to small molecules, and the Lubrol-treatment does not affect activity of glutamine synthetase. Evidence has also been obtained that indicates in vivo functioning of the cascade-control mechanism for adenylylation and deadenylylation of glutamine synthetase.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: E. coli glutamine synthetase is regulated by the covalent attachment and removal of 5'-AMP residues from each of the enzyme's 12 subunits. This adenylylation and deadenylylation process has been carefully studied in vitro. The primary objective of this project is to study the process in vivo, under conditions which are found physiologically. Permeabilized cells will be utilized for this study. These cells are made permeable to small molecules, but they retain larger molecules, including all enzymes required for functioning of the cascade system which regulates adenylylation.

Major Findings: Permeabilization of E. coli cells was the first goal of this study. This has been accomplished by treating the cells with Lubrol, a non-ionic detergent. Such treatment does not affect the activity of glutamine synthetase. Further, virtually all glutamine synthetase activity is retained within the permeabilized cells; there is no significant leakage into the incubation medium. Preliminary experiments also indicate that the complex cascade control of adenylylation and deadenylylation is functioning in the permeabilized cells. The state of adenylylation changes in response to alterations in concentration of glutamine and α-ketoglutaric acid, as predicted by the earlier in vitro studies.

It appears that p-chloromercuriphenyl sulfonate can inhibit the adenylyl transferase in permeabilized cells. This will allow direct assay of the state of adenylylation of glutamine synthetase within the cells.

Proposed Course of Research: The immediate objective is to identify and control variables which affect the permeabilization of the cells (e.g., pH). With permeabilization standardized, it will be possible to study in situ the various substances which are known to be effectors of the adenylylation/deadenylylation system in vitro.

Significance to Biomedical Research: An understanding of the mechanisms which regulate cellular metabolism would permit rational diagnosis and therapeutic intervention in numerous disease states. The adenylylation system is particularly important since it is a cascade control system which has been well-studied in vitro. The model which has emerged from the in vitro studies can now be confirmed or modified for the in vivo situation. The results will be relevant to other cascade systems, such as those of the coagulation and complement pathways.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00230-01 LB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Glutamine Synthetase in Pseudomonas

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

P.I.:	Jean-Marie Meyer	Visiting Fellow	LB NHLBI
Other:	E. R. Stadtman	Chief, Laboratory of Biochemistry	LB NHLBI

COOPERATING UNITS (if any)

None

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SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.8	0.2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Growth of Pseudomonas fluorescens on iron-deficient medium did not affect the levels of several enzymes presumed to be involved in the biosynthesis of the siderophore by this organism, including, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase, glutamate dehydrogenase, glutamine synthetase, and N^2-acetylornithine-5-aminotransferase. The level of glutamate synthase was considerably lower in iron-deficient cells as compared to iron-sufficient cells.

The concentration of glutamine synthetase in P. fluorescens was found to be 4-5 times greater than that found in Escherichia coli when grown under derepressing NH_4^+-limiting conditions. Although activity of the enzyme is regulated by an adenylylation cascade, metabolic regulation of this cascade appears to be different from that previously demonstrated in E. coli, since an unconventional pattern of adenylylation of the enzyme is observed when the organism is grown on either glycerol, succinate, malate, or fumarate. The glutamine synthetase from P. fluorescens has been purified to homogeneity and many of its properties have been determined.

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Project Objectives

(1) Examination of the level of enzymes in Pseudomonas during iron-limited conditions of growth in order to detect some enzymes perhaps involved in the biosynthesis of the siderophore of these bacteria.

(2) Purification, physiochemical properties, and regulation of activity of the glutamine synthetase of Pseudomonas fluorescens.

Major Findings

(1) The enzyme followed during the iron starvation studies were 3-deoxy-D-arabino heptulosonate 7-phosphate synthase (DAHP synthase) which occurs in the first step of the biosynthesis of aromatic compounds, glutamate dehydrogenase, glutamine synthetase, glutamate synthase, and N^2-acetyl-ornithine-5-aminotransferase which are involved directly or indirectly in the biosynthesis of ornithine and arginine. The choice of these enzymes was dictated by the fact that the siderophore excreted by P. fluorescens is, in part, constituted by an aromatic chromophore from the quinoleine family and a peptide chain containing basic amino acids such as δ-N-hydroxyornithine and/or arginine.

It has been shown that glutamate synthase is present in low levels when the cells are in an iron-deficient state compared with the amount present in iron-sufficient cells. This may indicate that this enzyme is an iron-containing protein, as it was shown in E. coli. However, the levels of the other enzymes tested were not affected by the iron content of the growth medium.

During this research, it was observed that the glutamine synthetase activity of P. fluorescens was very high compared with E. coli grown in the same conditions (4 to 5 times higher). Therefore, some experiments were begun which are the subject of the other topic of this report.

(2) Glutamine Synthetase of Pseudomonas fluorescens

Preliminary experiments have shown that the regulation of glutamine synthetase of P. fluorescens differs from that of the E. coli enzyme. In a synthetic medium, when nitrogen source is furnished in a nonlimiting amount as NH_4^+, E. coli glutamine synthetase is present in the cells in an almost fully adenylylated state. This adenylylated form is considered to be inactive within the cell. Under NH_4^+-limited conditions of growth, the enzyme is fully unadenylylated and active. In the case of P. fluorescens grown on NH_4^+-limited medium, the glutamine synthetase is present at a low state of adenylylation only at the beginning of the growth and reaches high values of adenylylation when the growth enters into the stationary phase. Conversely, in NH_4^+-sufficient medium, the state of adenylylation is very high during the exponential growth, but decreases drastically during the stationary phase. Such variations do not occur in the case of the E. coli enzyme.

These unexpected results and the very high activity found in derepressed cells (NH_4^+ starved cells) led us to purify the P. fluorescens glutamine synthetase in order to define the properties of this enzyme.

(a) Purification of Glutamine Synthetase of Pseudomonas fluorescens

The technique developed for the E. coli enzyme purification (Zn-Mg precipitation) gave poor results when applied to P. fluorescens enzyme. We developed a different way of purification which takes advantage of the low solubility of this enzyme in the absence of KCl. After heat treatment, $SO_4(NH_4)_2$ precipitation, and acetone precipitation, the enzyme, solubilized in imidazole 10 mM, $MnCl_2$ 10 mM, KCl 0.3 M buffer, was specifically precipitated by dialysis against the same buffer, but with KCl omitted.

The recovery of the enzyme activity during this procedure was between 50-60%. Purity was seen to be more than 95% by SDS gel electrophoresis.

(b) Molecular Weight

By SDS-PAGE electrophoresis, the molecular weight of the subunit of this enzyme appeared to be 62,000, a value higher than the E. coli subunit (50,000 MW). In fact, when both enzymes were run on the same gel, the two proteins were very well separated.

(c) Evidence for an Adenylylated State of the Enzyme

The possible presence of AMP in the glutamine synthetase molecule was expected from the results of several experiments. (1) The enzyme could be precipitated by anti AMP-BSA antiserum. (2) Treatment with snake venom phosphodiesterase resulted in a loss of activity of the glutamine synthetase (unadenylylated GS is less active than the adenylylated one, in the method of measurement we used.) (3) During phosphodiesterase treatment a soluble compound which absorbs at 260 nm was released. Identity of this compound is currently being established.

(d) Activity of Adenylylated and Unadenylylated Glutamine Synthetase In vitro

The activity of both forms of enzyme depended on the pH of the reaction mixture. From these studies, the inactivity point of the two forms was established. The value obtained, 8.35, is quite different than the value of the E. coli enzyme (7.57) under the same conditions.

Preliminary Conclusions

These results led to the following conclusions: the glutamine synthetase of P. fluorescens appears to be slightly different from the E. coli enzyme in the size of the subunit and the pattern of the activity versus pH; but it seems that the activity of the enzyme in the cell is regulated by the

same process of adenylylation as in E. coli. However, the preliminary physiological studies indicate that the response of the cell to different NH_4^+ levels by the adenylylation-deadenylylation process seems to be different in P. fluorescens than in E. coli.

The reason for this difference is still unknown. The carbon metabolism of this bacterium may be implicated. Indeed, these variations occurred when the bacteria was grown in succinate, fumarate, malate, or glycerol media, but do not occur when citrate or glucose are the carbon source for the growth.

Proposed Course of Action

Efforts will be directed to a more precise understanding of the mechanism of the regulation of glutamine synthetase in P. fluorescens and its physiological significance. Attempts will be made to see if the unusual properties of the regulation system of this enzyme, compared with the E. coli system, could be extended to the other Pseudomonas.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00231-01 LB

PERIOD COVERED
October 1, 1978 to September 30, 1979

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	NHLBI	LB
Others:	E. R. Stadtman	Chief, Laboratory of Biochemistry	NHLBI	LB
	P. Boon Chock	Research Chemist	NHLBI	LB
	Sue Goo Rhee	Research Chemist	NHLBI	LB

COOPERATING UNITS (if any)
None

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Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.4	1.1	0.3

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Two enzymes, a cyclic-AMP-dependent protein kinase and a phosphoprotein phosphatase, have been purified to near homogeneity from bovine heart. Meanwhile, histone H1 (or f1), which is phosphorylated by cyclic-AMP-dependent protein kinase at only one site, has been purified from rabbit thymus to serve as an interconvertible substrate. Kinetic experiments are being carried out to quantify the parameters which define a monocyclic cascade system.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To synthesize a well defined in vitro cyclic cascade system so that the kinetic equations and predictions developed in this laboratory, which describe such intracellular systems, can be tested. Phosphorylation and dephosphorylation of histone H1 has been chosen as a model for such systems.

Major Findings

None to date.

Proposed Course of Action

Now that all of the components necessary to build this monocyclic cascade system have been purified, experiments will be carried out to quantify the different kinetic variables and test the equations used to define a steady-state condition.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00232-01 LB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Biosynthesis of ^{75}Se-Transfer Nucleic Acids by Clostridium sticklandii

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

P.I.:	Ching-San Chen	Visiting Associate	LB NHLBI
Other:	Thressa C. Stadtman	Chief, Section on Intermediary Metabolism and Bioenergetics	

COOPERATING UNITS (if any)
None

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Section on Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.9	0.1

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Three tRNAs were labeled with ^{75}Se in cultures of Clostridium sticklandii incubated in the presence of $^{75}SeO_3^{=}$ and antibiotics that block either protein synthesis or DNA-dependent RNA synthesis. These tRNAs, tentatively called seleno-tRNAs I, II, and III according to their elution sequence from a benzoylated DEAE-cellulose column, were partially purified by chromatography on benzoylated DEAE-cellulose and DEAE-Sephadex A-50 columns. The ^{75}Se in these tRNAs was in the polynucleotide portion of the molecule and not in the form of esterified (alkali-labile) selenocysteine. The purified seleno-tRNA II could be aminoacylated with L-proline. The L-proline acceptor activity was cochromatographed with the seleno-tRNA II on the DEAE-Sephadex A-50 column. These results suggest that seleno-tRNA II is a selenium-containing L-prolyl-tRNA.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Protein A of Clostridium sticklandii glycine reductase complex contains selenocysteine which is essential for the biological activity of the protein. It was found that some macromolecules (tRNAs?) became labeled with ^{75}Se under conditions which both the biosynthesis of biologically active selenoprotein A and incorporation of ^{75}Se in the protein were inhibited by antibiotics that block either protein synthesis or DNA-dependent RNA synthesis. Since little is known about the biosynthesis of selenocysteine and the mechanism of incorporation of selenium into selenoprotein A, it is of interest to study the structure and function of the unknown ^{75}Se labeled molecules and their possible relationship with the formation of selenoprotein A.

Major Findings

(1) Three ^{75}Se labeled tRNAs, tentatively called seleno-tRNAs I, II, and III, were isolated from C. sticklandii cells incubated in the presence of $^{75}SeO_3$ and chloramphenicol by phenol extraction, adsorption, and desorption on a DEAE-cellulose column, and chromatographed on benzoylated DEAE-cellulose and DEAE-Sephadex A-50 columns. The purified seleno-tRNA II could be aminoacylated with L-proline. The L-proline acceptor activity was cochromatographed with the seleno-tRNA II on the DEAE-Sephadex A-50 column.

These results suggest that seleno-tRNA II is a selenium-containing L-prolyl-tRNA. The purified seleno-$tRNA^{Pro}$ accepted 109.2 pmole of L-proline per 1 A_{260} unit of the tRNA.

(2) A ^{75}Se labeled tRNA also was isolated from a clostridial species which ferments choline. The elution profile of this seleno-tRNA on a benzoylated DEAE-cellulose column indicated that this tRNA was different from those three seleno-tRNA species isolated from C. sticklandii.

Proposed Course of Research

The seleno-tRNAs I and III of C. sticklandii and the seleno-tRNA of the choline fermentor will be identified. The structure of the selenium-containing moieties of these four seleno-tRNA species also will be studied.

Publications

Stadtman, T. C., Dilworth, G. L., and Chen, C. S.: Selenium-Dependent Bacterial Enzymes, Abst. of the III International Symposium on Selenium and Tellurium Chemistry, France (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00233-01 LB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Menadione-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

PI:	J. N. Davis	Biochemistry	LB	NHLBI
OTHER:	T. C. Stadtman	Chief, Section on Intermediary Metabolism and Bioenergetics	LB	NHLBI

COOPERATING UNITS (if any)
None

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Section on Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION
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 ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A quinone-dependent phosphatase was previously purified to homogeneity from Clostridium sticklandii and its amino acid composition determined. The only known substrate for this phosphatase was p-nitrophenylphosphate. The recent observation that ^{32}P could be cleaved from [^{32}P] phosphocasein by this enzyme suggests that its natural substrate is a bacterial phosphoprotein. Hence a role in cellular regulation or in a phosphate transfer process is indicated. An affinity chromatographic procedure for direct isolation of the phosphatase from crude bacterial extracts was developed by covalently attaching a quinone analog (4-amino-2-methyl-1-naphthol) to Sepharose 4B.

PHS-6040
(Rev. 10-76)

Project Description

1. To isolate and identify the natural substrate of the bacterial p-nitrophenylphosphatase that has been purified to homogeneity from Clostridium sticklandii.

2. Test the ability of the purified phosphatase to interact with the glycine reductase system and serve as alternate acceptor for the phosphate that is esterified concomitant with glycine reduction. In the in vitro system ADP is the phosphate acceptor and ATP is formed. If such an interaction can occur 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.

3. Identify the natural quinone cofactor of the phosphatase which is replaced by menadione in vitro.

Major Findings

(1) Casein which had been phosphorylated with [γ^{32}P] by a protein kinase from yeast was tested as a possible substrate for the menadione-dependent p-nitrophenylphosphatase from C. sticklandii. Labeled phosphate was released from the [^{32}P] casein in the presence of the phosphatase in a time and menadione dependent reaction. Since the casein substrate was known to contain the phosphate in ester linkage to serine and threonine residues, these results suggest that peptides containing O-phosphoserine or O-phospho threonine might be the natural substrate of the phosphatase.

(2) An affinity chromatography method was devised to uniquely separate the quinone-dependent phosphatase from other non-specific phosphatases in extracts of C. sticklandii. For this purpose advantage was taken of the affinity of the phosphatase for 4-amino-2-methyl-1-naphthol (vitamin K_5). This menadione analog was covalently attached through the amino group to Sepharose 4B. The menadione-dependent phosphatase in extracts formed a complex with the ligand and was subsequently removed with 20 mN TES buffer containing 50 mM NaCl.

(3) A variety of other anaerobic bacteria were screened for the presence of the quinone-dependent phosphatase using the affinity column described above. The phosphatase could be isolated from crude extracts of C. sticklandii by this procedure but not from related amino acid fermenting organisms, e.g. Clostridium sporogenes and Clostridium lentoputrescens, or from Clostridium kluyveri.

Proposed Course of Action

(1) Attempts will be continued to identify the natural substrate of the menadione-dependent phosphatase. Other well-characterized phosphoproteins will be tested in order to determine the degree of specificity for the hydrolyzable phosphase ester linkage.

(2) The selenoprotein of the glycine reductase complex is considered a likely component of the system that might undergo phosphorylation during the reduction reaction and further attempts will be made to phosphorylate this protein either chemically or enzymically. An S-phosphocysteine residue on this protein is chemically reasonable and might serve as a substrate for the phosphatase. If so it would suggest a transfer role for the phosphatase in which the phosphate ester generated during glycine reduction would be used directly rather than after transfer to ADP to form ATP.

(3) Another approach that will be used to look for the phosphatase natural substrate will be to prepare phosphatase free extracts of C. sticklandii by passage over the affinity column and then to label these with [^{32}P] ATP enzymatically. The radioactive protein fractions separated from such preparations will then be tested as substrates for the purified phosphatase.

(4) Extracts of other microorganisms will be examined for presence of the phosphatase using the affinity column.

Keywork Descriptors

Menadione, Quinone-dependent phosphatase, sulphydryl enzyme, Clostridium sticklandii

Honors and Awards - None

Publications

E. R. Stadtman, P. Z. Smyrniotis, J. N. Davis, and M. E. Wittenberger: Enzymic Procedures for Determining the Average State of Adenylylation of Escherichia cole Glutamine Synthetase. Analytical Biochemistry, 95, 275-285 (1979)

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00234-01 LB

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
Attempts to Determine if Protein B of Glycine Reductase from Cl. sticklandii has a Pyruvoyl Component

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

P.I.:	Sue H. Neece	Chemist	LB NHLBI
OTHER:	Thressa Stadtman	Chief, Section on Intermediary Metabolism and Bioenergetics	LB NHLBI

COOPERATING UNITS (if any)

Murray Cohn LBP NIAMDD

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NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.25	0.2	0.05

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Protein B of glycine reductase from Cl. sticklandii apparently has a pyruvoyl component. This was evidenced by co-chromatography of tritiated material from borotritide-reduced and acid-hydrolyzed protein B with standard ^{14}C-lactic acid on Aminex A27.

PHS-6040
(Rev. 10-76)

Project Description: Project No. Z01 HL 00234-01 LB

Objective: Tanaka and Stadtman found previously that pre-treatment of protein B with borohydride or hydroxylamine inactivated it in the glycine reductase assay. This indicated the presence of a carbonyl group and since pyridoxal phosphate is not a cofactor, this project was undertaken to determine if a pyruvoyl group is present.

Major Findings: Reduction of protein B with tritiated borohydride followed by acid hydrolysis, lyophilization and extraction with methanol produced tritiated material which co-chromatographed with standard ^{14}C-lactic acid on the highly resolving ion exchange resin, Aminex A27.

Proposed Course of Action: Further characterization of the tritiated material will be undertaken using gas chromatography, high pressure liquid chromatography, thin layer chromatography and testing as a substrate in the lactic dehydrogenase assay.

Also the material will be quantitated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00235-01 LB

PERIOD COVERED
October 1, 1978 to September

TITLE OF PROJECT (80 characters or less)
Properties of the 5-Deazaflavin-Dependent $NADP^+$ Reductase 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
Other:	Thressa C. Stadtman	Chief, Section on Intermediary Metabolism and Bioenergetics	LB	NHLBI
	Lin Tsai	Research Chemist	LB	NHLBI

COOPERATING UNITS (if any)
None

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NHLBI, NIH, Bethesda, Maryland 20205

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1.6	1.3	0.3

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

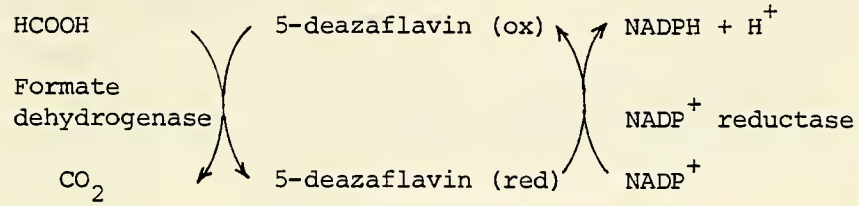
A rapid and highly sensitive fluorometric assay for the 5-deazaflavin-dependent $NADP^+$ reductase was developed, and the reductase was purified and characterized.

The structural requirements of the 5-deazaflavin cofactor were studied using the purified reductase and synthetic analogs.

PHS-6040
(Rev. 10-76)

Project Description

The 5-deazaflavin-dependent $NADP^+$ reductase from Methanococcus vannielii participates in the following overall reaction:



The aim of this project is to characterize the 5-deazaflavin-dependent $NADP^+$ reductase and study structural requirements of the 5-deazaflavin cofactor by testing synthetic analogs as substrates for the reductase.

Major Findings

A rapid and highly sensitive assay for the 5-deazaflavin-dependent $NADP^+$ reductase was developed without the necessity of coupling it to formate dehydrogenase (an extremely oxygen-sensitive enzyme). The initial rate of the reaction of 5-deazaflavin with NADPH was followed by measuring the decrease of the emission at 475 nm when the assay mixture was irradiated at 425 nm. Similarly, the reverse reaction was assayed by observing the increase of fluorescence intensity.

The reductase was purified to homogeneity by chromatography on agarose-$NADP^+$ affinity column, DEAE-cellulose and Sephacryl S-200. The molecular weight was estimated to be 85,000 daltons by Sephacryl S-200 gel filtration. The reductase has a subunit of 22,000 daltons estimated by SDS gel electrophoresis. The enzyme is probably a tetramer composed of four apparently identical subunits. Inhibition by iodoacetamide and reversible inhibition by p-chloromercuriphenyl sulfonic acid indicated involvement of sulfhydryl group(s). The reductase has pH and temperature optima at pH 4.6 and 20° C.

The reductase in the presence of excess NADPH can reduce synthetic analogs of 5-deazaflavin cofactor, but not FAD, FMN, riboflavin or 5-methyl-5-deazariboflavin. This result indicated that the 5-position of the aromatic chromophore has to be carbon (not nitrogen) and unsubstituted. Comparison of kinetic data of 5-deazariboflavin, 8-hydroxyl-8-desmethyl-5-deazariboflavin, and 8-hydroxyl-7,8-didesmethyl-5-deazariboflavin suggested that the 8-hydroxyl group and unsubstituted 7-position are also important in the structural requirements of the 5-deazaflavin cofactor.

Km and Turnover Number of the 5-Deazaflavin Cofactor and Synthetic Analogs

$$\text{5-deazaflavin (ox)} + \text{NADPH} + H^+ \longrightarrow \text{5-deazaflavin (red)} + NADP^+$$

Substrate	Km	Turnover number
	μM	min^{-1} per molecule
Natural 5-deazaflavin cofactor	2.8	442
8-Hydroxyl-7,8-didesmethyl-5-deazariboflavin [a]	3.1	174
8-Hydroxyl-8-desmethyl-5-deazariboflavin [a]	3.2	11
5-Deazariboflavin	6.9	4.5

[a] These compounds were a generous gift from Professor C. Walsh (Massachusetts Institute of Technology).

Proposed Course of Action

(1) The stereochemistry and the mechanism of the reaction catalyzed by 5-deazaflavin-dependent $NADP^+$ reductase will be studied.

(2) The biosynthesis of the natural 5-deazaflavin cofactor in M. vannielii will be studied.

Publications

Yamazaki, S., Stadtman, T. C., and Tsai, L: Properties of 5-Deazaflavin-Dependent $NADP^+$ Reductase from Methanococcus vannielii. Abst. XIth International Congress of Biochemistry, Toronto, Canada, July 8-13, 1979.

Yamazaki, S., Tove, S. B.: Biohydrogenation of Unsaturated Fatty Acids. Presence of Dithionite and an Endogenous Electron Donor in Butyrivibrio fibrisolvens, J. Biol. Chem. 254, 3812-3817, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00236-01 LB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Control of Enzyme Degradation

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

P.I.:	Michael R. Maurizi	Individual National Research Service Awardee	LB	NHLBI
Other:	E. 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:	PROFESSIONAL:	OTHER:
1.4	1.1	0.3

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Project A - The inactivation of phosphofructokinase in extracts of Tetrahymena pyriformis cells is reversible. Loss of activity does not appear to involve phosphorylation of the enzyme.

Project B - The rates of loss of specific enzyme activities during starvation of Escherichia coli cells show considerable variation. The relative rates of inactivation of the various enzymes are different depending on whether glucose or ammonia is limiting. There are large differences in the rates of decline of some enzyme activities when different strains of E. coli are starved under similar conditions.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: General -- The aim of this work is to establish an in vitro or in situ system for the study of specific enzyme inactivation and degradation. The identification and characterization of the components of the degradative system and the elucidation of the mechanism and control of the system will then be attempted.

Specific: Project A. Research was conducted to define the biochemical basis of the inactivation of phosphofructokinase in extracts of Tetrahymena pyriformis (P. W. Royt, Annual Report, 1976-77) and to identify the extract components required for the inactivation. The possibility that inactivation involved degradation of the enzyme or was followed by degradation of the enzyme was a primary consideration. Project B. Protein degradation has been studied in several laboratories, but most studies have not determined the differential rates of degradation of specific enzymes and proteins. This project designed to identify those native proteins that are most rapidly degraded in starving E. coli cells and to establish systems for the study of the mechanism and control of that degradation.

Major Findings

Project A

(a) Distribution of Phosphofructokinase (PFK). In sonic extracts of T. pyriformis, 20-80% of PFK is found in the soluble fraction. This activity is labile and disappears rapidly in the absence of stabilizing solvents (20% glycerol or DMSO). The membrane-bound PFK is relatively stable and cannot be solubilized by sonication or by moderate (0.5 M) salt washes. Soluble PFK is inactivated at 30° C when incubated with ATP, fluoride, magnesium, and the endogenous soluble factor in the extracts. This inactivation requires higher concentrations of the soluble factor than does inactivation of membrane-bound PFK.

(b) Requirements for Inactivation. Fluoride (usually 20 mM) is required for inactivation of PFK. At 2 mM fluoride, inactivation is very slow, but goes nearly to completion in 1 hour. Fluoride at 20 mM partially inhibits the high ATPase activity in membrane preps of PFK, but it is likely that fluoride has another function in the inactivation as well. Soluble preps of PFK have lower ATPase activity and it is possible to obtain complete inactivation of PFK at much lower ATP levels (0.5 mM). Even at high ATP (10 mM), however, fluoride is still required for inactivation of soluble PFK. When fresh membrane preps of PFK are used, inactivation requires a protein from the soluble extract (P. W. Royt), and the rate of inactivation is proportional to the amount of factor used. Older membrane preps -- ones that have been kept on ice for long periods, frozen and thawed, or incubated at 30° C for 1 hour -- can be inactivated without addition of soluble factor. Preincubation of fresh membrane-bound PFK with ATP results in a more rapid inactivation upon addition of fluoride, magnesium, and soluble factor. EDTA (10 mM) and phosphate (250 mM) block inactivation. Fructose-6-phosphate has no effect on

activation.

(c) Reactivation of Inactive PFK. Completely inactivated PFK can be reactivated by dilution (10-30-fold) and incubation at 30° C (10-60 minutes). Removal of inactivating factors on Sephadex G-25 leads to only partial reactivation without further dilution and incubation at 30° C. The requirement for incubation at 30° C suggests a slow release of an inhibitor or a slow conformational change in the enzyme. ATP stimulates the rate of reactivation and improves the yield. Fluoride (20 mM) partially blocks reactivation. The substrate, fructose-6-phosphate, completely prevents reactivation. Dilution of inactive PFK with an equal volume of 10 mM EDTA leads to a rapid return of activity (5 minutes). Both soluble and membrane-bound PFK can be reactivated.

(d) Properties of Inactive PFK. Membrane-bound PFK is not solubilized during inactivation. Membrane preps of PFK can be inactivated, isolated by ultracentrifugation, washed, and reactivated with high yield. The molecular weight of soluble PFK does not change during inactivation as judged by reactivation of inactive PFK run on an AcA34 gel filtration column. Inactivation of PFK in the presence of γ^{32}P-ATP gave no evidence for the incorporation of phosphate into PFK.

Project B

(a) Enzyme Levels during Starvation. Protein degradation increases in E. coli during starvation. A previous study in this laboratory (D. M. Pehlke, Annual Report, 1973-74) examined the fates of 12 enzymes during glucose and ammonia starvation. This work extends those studies. During nitrogen starvation, the activities of several enzymes declined more rapidly (> 10%/hour) than the reported rates of general protein turnover (3-4%/hour) [glutamate dehydrogenase (GDH), deoxyarabino-heptulosonate-phosphate synthetase, ornithine transcarbamylase (OTCase), and aspartokinase-III (AKIII)]. Activities of malate dehydrogenase, aspartokinase I, glutamate synthase, and aspartate transcarbamylase (ATCase) declined more slowly (4-8%/hour). Activities of the following enzymes either increased or remained constant: α-ketoglutarate dehydrogenase, phosphofructokinase (PFK), aldolase, malic enzyme, phospho(enol)pyruvate synthase, glutamine synthetase, glucose-6-phosphate dehydrogenase (G6PDH), and 6-phosphogluconate dehydrogenase (6PGDH). During glucose starvation, the activities of GDH, ATCase, OTCase AKIII, and glutamate synthase declined, but more slowly than during nitrogen starvation. The activities of glutamine synthetase, PFK, aldolase, G6PDH, and 6PGDH remained constant or increased slightly. These studies measured only the net change in enzyme levels, and it is possible that some of the activities measured represent the sum of continued synthesis and inactivation. However, the addition of uracil (to repress synthesis of ATCase) or lysine (to repress AKIII) had little effect on the apparent rates of inactivation of the respective enzymes suggesting that resynthesis of these two enzymes is not occurring during starvation.

It has not been demonstrated that the loss of any of these enzyme

activities results from proteolytic degradation. Addition of chloramphenicol to ammonia-starved cells, however, did prevent the loss of OTCase, GDH, and AKIII), but not glutamate synthase. Since chloramphenicol is known to block protein degradation in starving E. coli this result is consistent with a proteolytic mechanism for the former inactivations.

(b) Further Studies with GDH and AKIII. Addition of ammonia to starving cells results in new growth and reappearance of GDH and AKIII. Growth and return of the enzyme activities are completely blocked by chloramphenicol. Since recovery of GDH and AKIII requires protein synthesis, the initial loss of activities during starvation most likely was due to degradation.

In an effort to optimize conditions for inactivation, cells were starved for ammonia in the presence of various carbon sources. Inactivation of GDH and AKIII were very similar whether glucose, gluconate, glycerol, succinate, or acetate was the carbon and energy source. When GDH and AKIII inactivations were followed in different strains of E. coli, significant differences were observed. Both enzymes were stable in E. coli strains B, 15, and 3000 (< 1%/hour). Inactivation was significant in strains K12 and N99 (10-20%/hour) and very rapid in strains C and W (20-40%/hour).

Relevance to Biomedical Research

Metabolic regulation is accomplished in part by control of enzyme levels which in turn are determined by the sum of synthesis and degradation of the enzymes. Clearly, the degradation of enzymes must be regulated since uncontrolled degradation would be harmful and wasteful. It is known that alterations in the degradative systems are part of the physiological consequences of starvation and aging and of such diseases as diabetes and muscular dystrophy. In addition, selective degradation of proteins may be important during viral infections and during cellular differentiation.

In most cases the degradative systems of cells are incompletely understood. Very little is known concerning the initiation and control of degradation of individual enzymes. Since protein turnover in E. coli shares at least two important features with turnover in mammalian cells, i.e., selectivity and energy dependence, E. coli is a convenient model system in which to study this process.

Proposed Course of Action

Project A

The inactivation of PFK in Tetrahymena extracts may reflect interesting properties of this enzyme, but does not involve proteolysis. No further work on this project is planned for the present time.

Project B

In vivo studies. Extending the survey of enzymes and proteins that are

degraded during starvation will help define the scope of the degradative system of E. coli and should identify suitable substrate proteins for use in in vitro reconstructions of the degradative process. Further definition of the conditions that lead to rapid degradation will be attempted. The effect of factors that influence "general protein" and "abnormal protein" turnover on the degradation of specific enzymes will be tested. For this purpose the examination of mutant strains (e.g., deg^-, rel^-, cat^-) will be useful. In addition to following enzyme activities, the fate of enzyme protein will be monitored by immunochemical techniques. Antibodies to AKIII have been prepared and antibodies to other enzymes can be obtained if warranted.

In vitro studies. A major goal will be the reconstruction of an in vitro system that mimics the in vivo degradative system. Several approaches are possible: (a) initial efforts will be directed toward determining whether covalent modification precedes degradation of AKIII as has been suggested by Westhead (personal communication); (b) at least four proteases have been isolated from E. coli. Selected enzymes can be tested in in vitro systems in which one or more of these protease activities are optimized. The effects of prior modification or ligand binding on the degradation of specific enzymes will be followed; (c) the susceptibility of select enzymes to the nonproteolytic inactivation system described by R. Levine (Annual Report, 1979) can be investigated.

For all of the above approaches, the use of strains that do not inactivate or degrade the enzymes under study may provide useful controls and contribute in other ways to these analyses.

ANNUAL REPORT OF THE CARDIOLOGY BRANCH
National Heart, Lung, and Blood Institute
October 1, 1978 through September 30, 1979

The basic areas of experimental interest of the Cardiology Branch relate to the pathogenesis, pathophysiology and treatment of coronary artery disease; molecular mechanisms responsible for the contractile function of the heart; development of noninvasive techniques to assess cardiac structure and function; and the application of multidisciplinary techniques to define the determinants of irreversible heart failure in patients with valvular heart disease. During the past two years emphasis has been placed on studies designed to define the risk factors predictive of sudden death in patients 1) with stable coronary artery disease 2) following an acute myocardial infarction, 3) with hypertrophic cardiomyopathy, and 4) with valvular heart disease.

CORONARY ARTERY DISEASE

Pharmacologic Treatment of Acute Myocardial Infarction (AMI)

We previously hypothesized that the platelet may play a role in AMI in that acute ischemia may stimulate local aggregation of platelets, which in turn would interfere with coronary collateral flow (CCF). Last year we found that aspirin, a potent inhibitor of platelet aggregation, significantly increased CCF four hours following acute occlusion of the left anterior descending coronary artery in dogs. Since recent studies suggested that daily administration of sulfinpyrazone (SPZ) decreases patient mortality during the first year following acute AMI, and since SPZ inhibits platelets, we determined whether SPZ, like ASA, has the potential to increase CCF in dogs subject to acute occlusion of the left anterior descending (LAD) coronary artery. Four hours following LAD occlusion no significant changes in CCF occurred in control dogs when compared to CCF measured 5 min after occlusion. However, in the ischemic zone epicardium of the SPZ treated animals, CCF rose from 0.13 ml/min/g 5 min after occlusion to 0.31 ($p<.01$) 4 hrs after occlusion. Thus, SPZ, like ASA, can increase CCF to ischemic myocardium. This favorable effect may play a role in the salutary action of SPZ in patients following AMI.

To determine the conditions under which increases in CCF can lead to reduction in infarct size, a model of AMI in dogs was developed to determine total mass of myocardium salvaged by an intervention during acute coronary occlusion. We studied the effects of three drugs: ASA and SPZ, both potent platelet inhibitors shown previously to increase CCF during acute coronary occlusion, and naproxen (NAP), also a platelet inhibitor but a drug that has greater lysosomal stabilizing activity. Each drug was administered in doses that markedly inhibited platelet aggregation prior to and during occlusion of the LAD. Dogs were sacrificed 72 hours after occlusion and analyzed for mass of myocardium that became necrotic. None of these drugs

altered infarct size. These results suggest that while ASA and SPZ increase CCF in experimental acute coronary occlusion (an effect that may be responsible for the acute beneficial effects of ASA and SPZ on experimental arrhythmias during acute infarction, 1) platelet inhibition, per se, does not affect ultimate infarct size, and 2) the magnitude of increase in CCF (25-50%) is not sufficient to reduce infarct size.

A separate study, similar to the above, was carried out with nitroglycerin, a drug we have shown 1) in experimental AMI reduces the incidence of ventricular fibrillation and the damage occurring after release of occlusion following 5 hrs of ischemia, and 2) in patients with AMI and left ventricular failure results in favorable hemodynamic and electrophysiologic effects. In the present study, in which coronary occlusion was sustained and dogs were not in LV failure, NTG was effective in reducing infarct size only in those dogs with small areas at risk of infarction (less than 35% of LV). NTG did not alter infarct size in those dogs with larger areas at risk. These results suggest that 1) size of area at risk may importantly influence drug effect, and 2) in the absence of overt LV failure, NTG may only reduce the size of small infarcts.

Our current plans are to determine whether 1) NTG reduces the size of large infarcts when LV failure is present and 2) interventions acting through different mechanisms can work synergistically and thereby more effectively reduce infarct size.

Biochemical, ultrastructural and immuno-histological evidence suggests that lysosomal hydrolysis may play a causal role in death of myocardial cells that otherwise may 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. We therefore determined the rate of proteolysis following acute LAD occlusion in dogs. This was assessed by determining the amount of tyrosine produced by ischemic and non-ischemic slices of myocardium (in the presence of cycloheximide, which blocks protein synthesis, the production of this amino acid is an index of the rate of protein breakdown). We also assessed the effects of leupeptin, an inhibitor of thiol-proteases (including lysosomal cathepsin B). Although leupeptin produced the expected decrease in proteolysis, our initial results suggest (surprisingly) that ischemia leads to a <u>decrease</u> in proteolysis. If further studies substantiate these preliminary results, it would appear unlikely that ischemia-induced release of lysosomal enzymes leads to premature myocardial necrosis and that interventions capable of stabilizing lysosomal membranes will thereby preserve ischemic myocardium.

Diagnosis and Treatment of Stable Coronary Artery Disease (CAD)

Indications for coronary artery bypass grafting (CABG) in pts with stable CAD
Attitudes regarding indications for CABG in pts with stable CAD are based on natural history studies derived largely from severely symptomatic patients studied in the late 1960s and early 1970s. These data indicate an approximately 6 to 11% annual mortality in pts with 2 or 3 vessel disease. Post-op mortality averages about 2 to 5% per year. This comparison yields a compelling argument for operating on all pts with extensive CAD. However, during the past four and one half years we have been evaluating pts with CAD who are asymptomatic or have only mild symptoms. To date 95 pts have been followed for over 2 yrs: 73% had stenoses of 2 or 3 coronary arteries; LV function was normal in 65% of pts. Overall annual mortality is only 1.5%, and 2% in pts with 2 or 3 vessel disease. These new natural history data will importantly affect operative strategy for CAD pts. Given such results, it is highly unlikely that prophylactic CABG could enhance survival of the asymptomatic or moderately symptomatic pt.

The search for left main CAD CABG prolongs life in pts with left main CAD. Hence, it is imperative to determine the prevalence of left main CAD in various subgroups of pts with CAD and to identify such pts non-invasively. In 300 consecutive pts with CAD 140 were in functional Class I or II and 160 were in functional Class III or IV. Left main CAD was present in 10% of Functional Class I and II pts and 16% in more severely symptomatic pts. 50% of CAD pts with no or mild symptoms had $\geq$1 mm ST segment depression during graded exercise, 20% of whom had left main CAD. Only 7% of pts with left main CAD had a normal ST segment response to exercise. Hence, exercise screening may be employed to select patients who should have coronary angiography to determine whether they are operative candidates (i.e., have left main CAD), despite mild symptoms. Such screening for left main CAD decreases the number of patients who require angiography by half and fails to detect only about 10% of pts with left main CAD.

Identification of high risk pts following AMI Pts recovering from AMI are at relatively high risk of sudden death during the first six mos after discharge from hospital. More accurate identification of high risk pts might permit selection of pts for aggressive medical or surgical therapy to prolong life. We studied 45 pts one day prior to hospital discharge (2-4 wks after AMI) and again 6 to 13 mos later. Ejection fraction (EF) by radionuclide cineangiography correlated significantly with frequency and complexity of ventricular arrhythmias detected on 24-hr ECG tapes but neither exercise ECG ST segments nor plasma norepinephrine concentration (at rest or with exercise), correlated with EF or with arrhythmia. Four of the 45 pts died within one year of infarction; each had EF <35% at rest at early study, but neither presence of complex arrhythmias nor determination of EF during exercise provided additional help in predicting those who died. These results suggest that the high risk of sudden death in the first year following AMI is associated with poor LV function. This is discouraging, since in such pts it is less likely aggressive medical or surgical treatment will appreciably alter outcome.

NHLBI Type II Coronary Intervention Study The primary aim of this randomized, double blinded, prospective study, carried out in collaboration with the Molecular Disease Branch, is to determine whether lowering LDL cholesterol with diet and
cholestyramine in pts with premature coronary artery disease
and Type II hypercholesterolemia will retard the progression of coronary artery disease. 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 yrs of treatment. Over one half the participants will have completed the study by Ocotber 1979. The study will be finished by 1981.

HYPERTROPHIC CARDIOMYOPATHY (HCM)

Natural history of pts with HCM Sudden death is not an uncommon occurrence in pts with HCM. Over the past few years we have been attempting to document the circumstances under which sudden death occurs, as well as elucidate predictors of sudden death so that more effective therapy might be instituted. We recently completed a study in which the clinical and pathologic features of 25 patients in whom sudden death was the first manifestation of disease were studied. The large majority of these patients demonstrated marked thickening of the ventricular septum and a distinctly abnormal electrocardiogram. In a separate study of 29 competitive athletes (ages 14-30 yrs) who had died suddenly and unexpectedly, hypertrophic cardiomyopathy was the cause of death in almost 50%, most of whom also had a relatively thick ventricular septum. Thus, HCM is a common (and often unrecognized) cause of sudden death in young, healthy, vigorous individuals. These, as well as previous studies in our laboratory, suggest that a thick ventricular septum and abnormal electrocardiogram are highly sensitive predictors of sudden death. However, because the specificity of such findings is low, additional studies are needed to better define the patient at risk of sudden death.

Distribution of the histologic abnormalities in HCM The most characteristic histologic feature of hearts with HCM is the presence of numerous disorganized cardiac muscle cells in the disproportionately thickened ventricular septum (VS). We previously found that cellular disorganization is ucommon in pts with diseases other than HCM and, when present, usually involves less than 5% of the VS. Most pts with HCM show marked cellular disorganization, usually involving 50% or more of the VS. These observations have been extended: we found disorganization to be marked not only in the VS, but also in the LV free wall in pts with HCM. Minimal disorganization of LV free wall was found in pts without HCM. Most marked degree of LV free wall and septal disorganization was in 15 pts with HCM (<25 yrs of age) in whom sudden death was the initial manifestation of heart disease. Hence, in most pts with HCM, cellular disorganization is diffusely distributed in both VS and LV free wall, and probably represents extensive distribution of a cardiomyopathic process.

Distribution of hypertrophy in pts with HCM M-mode echo has shown asymmetric septal hypertrophy of the VS to be a consistent feature of HCM. However, to better characterize distribution of hypertrophy throughout the LV wall, 74 pts with HCM were studied by wide-angle 2-dimensional (2D) echo. In 46 of the 74 pts hypertrophy was marked and diffuse, involving LV free wall and/or VS; these pts were compared to 28 with mild, localized hypertrophy. Moderate to severe symptoms were more common with diffuse (83%) than with localized hypertrophy (21%, $p<0.001$). LV outflow obstruction was also more common with diffuse (63%) than with localized hypertrophy (14%, $p<.001$). Thus, with 2D echo we found 1) hypertrophy in pts with HCM manifests a variety of patterns and wider distribution than has been appreciated by M-mode echo; 2) hypertrophy is asymmetric and often involves antero-lateral LV as well as VS; 3) functional limitation and outflow obstruction are most common in those pts with marked and diffuse hypertrophy.

We also employed this technique to study a group of 20 relatives of pts with HCM in whom HCM was suspected because of an abnormal ECG but could not be confirmed because of a normal M-mode echo. In 90% of these pts 2D echo exhibited localized areas of LV wall hypertrophy involving regions of myocardium through which the M-mode beam does not usually pass. Hence, 2D echo is sometimes necessary to identify HCM pts with unusual distribution of cardiac hypertrophy.

New approach to the pharmacologic treatment of HCM Although propranolol is effective in the treatment of the majority of pts with HCM, many are unresponsive. The only alternative therapy in pts with obstruction is operation, which carries a 5 to 10% risk. No alternative is available to those individuals without obstruction. We hypothesized that myocardial intracellular calcium overload may be a possible etiologic mechanism responsible for the hemodynamic abnormalities of pts with HCM. To test this hypothesis we are investigating the effects of verapamil, a calcium antagonist. In our first study we found that verapamil significantly decreases LV outflow tract obstruction both under control conditions and when the gradient is provoked by several interventions: basal gradient decreased from 84 to 56 mmHg and gradient provoked in response to amyl nitrite decreased from 82 to 43 mmHg. We then assessed the clinical implications of this finding by determining treadmill exercise capacity and symptomatic status in 19 pts with HCM during oral verapamil, propranolol and placebo, administered in randomized double-blinded fashion. Exercise duration increased with both drugs: 27% on verapamil and 22% on propranolol. Because of these results we have since discharged 38 pts on chronic oral verapamil, 15 of whom to date have had late exercise testing. Long-term verapamil led to improved exercise duration compared to in-hospital (a) control (+63%; $p<.001$), (b) propranolol (+51%, $p<.025$), (c) verapamil (+36%, $p<.005$).

Previous studies in our laboratory identified arrhythmias, both ventricular and supraventricular, as potentially important mechanisms responsible for sudden death in pts with HCM. Because of its potential antiarrhythmic properties and the beneficial hemodynamic effects observed in pts with HCM, verapamil has been administered to 15 pts with clinically significant arrhythmias; 10 supraventricular and 5 ventricular. We found verapamil to

be very effective in controlling atrial tachyarrhythmias. This observation is especially relevant to those HCM pts with chronic AF who previously required digoxin to slow the ventricular response, a drug that also increases LV outflow tract obstruction. Verapamil does not appear to be as consistently helpful in ventricular arrhythmias, although it is not without benefit; of 5 pts with frequent VPCs, 2 showed greater than 75% reduction in frequency following verapamil.

We conclude that verapamil 1) decreases LV outflow tract gradient, 2) improves exercise capacity and 3) is effective in the control of certain arrhythmias. Although verapamil appears to offer a much-needed new therapeutic approach to the treatment of HCM, the drug is not without adverse effects. About one -third of patients are unable to tolerate the drug or do not respond to it. In addition, 2 pts died while on verapamil therapy. Thus, additional studies are necessary to determine which patients will respond to the drug and which might experience serious complications.

VALVULAR HEART DISEASE

Functional alterations of the LV to chronic aortic regurgitation (AR) To assess the relation of LV volume overload to LV systolic function in chronic AR, 80 pts were studied by both echo and radionuclide cineangiography (RN) at rest and during exercise. LV fractional shortening remained within the normal range until LV diastolic dimension by echo exceeded 65 mm and LV systolic dimension exceeded 42 mm (both representing 140% of expected normal values). Above these dimensions, fractional shortening fell progressively. Similarly, LV EF at rest and during exercise by RN was normal in pts with LV dimensions not exceeding 140% of normal. Above this value, EF was often normal at rest, but was subnormal during exercise in virtually all pts. Thus, the LV can increase its internal dimensions approximately 40% and maintain normal systolic function. When this point is exceeded, further dilatation is accompanied by progressive LV systolic dysfunction.

Elucidation of the determinants of irreversible myocardial failure
If operation for pts with AR is deferred until significant symptoms develop, close to 50% die within 5 yrs of operation. Most die from CHF secondary to irreversible myocardial damage. To determine whether any pre-op parameters accurately predict high risk of late post-op death, 49 pts undergoing aortic valve replacement for AR were prospectively analyzed. There were three op deaths (6%) and 8 pts died of CHF 5 to 37 mos following op. Echo determinants of systolic function (EF, fractional shortening, and end systolic dimension) were highly correlated with late CHF death. Thus, 17 pts preop had LV end systolic dimension >55% mm. Of these, 53% died either at op or of CHF late post-operatively. In contrast, of 32 pts with end systolic dimension <55, only 2 deaths (6%) occurred, either at op (1) or due to CHF (1). These results suggested that pts should be operated upon once systolic dysfunction is documented, regardless of symptomatic status. The validity of this conclusion, however, depended on the unproven asumption that asymptomatic pts develop symptoms requiring operation relatively soon after appearance of systolic dysfunction.

Determining optimal time for operation To determine the validity of the above hypothesis, we studied prospectively 37 asymptomatic pts with AR. Mean follow up was 34 mos. Symptoms that required op developed in 14 pts (36%). The others remained asymptomatic during follow-up. Indices of LV systolic function were the most sensitive for distinguishing on initial examination pts who subsequently required op. While 4 of 5 (80%) pts with end systolic dimension >55 mm developed symptoms and came to operation only 4 of 20 pts (20%) with systolic dimension <50 mm developed symptoms that required operation, and none died during follow-up. Moreover, exercise studies demonstrated that pts with impaired systolic function on echo, but with good treadmill exercise capacity, have reversible myocardial disease; systolic function returned to normal after operation and death from CHF was rare. On the other hand, pts with impaired systolic function on echo who exercise poorly on the treadmill have a high incidence of impaired LV function postop and death from CHF is common. Thus, asymptomatic pts with AR whose end systolic dimension is <50 mm appear to be at low risk of developing irreversible LV failure and can be followed safely with echos at about yearly intervals. Asymptomatic pts with end systolic dimension between 50 and 54 mm are being followed with serial echos every 4 to 6 mos. Op is now being recommended to pts with end systolic dimensions of 55 mm or greater, even in the absence of symptoms.

Effect of exercise conditioning on plasma high density lipoprotein (HDL) Epidemiologic studies have demonstrated an inverse correlation between HDL cholesterol and incidence of CAD. Although physically active individuals tend to have higher HDL levels than their sedentary peers, it has yet to be shown that physical activity by itself can raise HDL when other variables remain constant. We examined the effect of a 6 week exercise conditioning program on 10 young normal subjects who were maintained on a constant composition iso-weight diet. Total cholesterol decreased significantly in those individuals who, prior to training, had total cholesterol greater than 200 mg/dl; there was no significant change in triglycerides, VLDL or LDL. HDL cholesterol decreased insignificantly from 47 to 43 mg/dl, and total HDL and HDL subfractions were unchanged. Thus, under the conditions of this study in which diet and weight were controlled, exercise conditioning per se did not elevate HDL.

MOLECULAR CARDIOLOGY

The section on Molecular Cardiology has conducted research on the biochemistry of the contractile and regulatory proteins isolated from human blood platelets, bovine brain, turkey gizzard smooth muscle, and canine hearts.

Smooth Muscle Contractile Proteins Phosphorylation of smooth muscle myosin by the specific enzyme, myosin light-chain kinase, is required for actin-activation of smooth muscle myosin ATPase activity. Myosin light-chain kinase has been purified to homogeniety as determined by gel electrophoresis under denaturing and non-denaturing conditions and found to have a molecular weight of 130,000 daltons. The enzyme is entirely inactive in the absence of a 16,500 dalton Ca^{2+}-binding protein (calmodulin) Ca^{2+}.

Myosin light chain kinase can be phosphorylated by the catalytic subunit of cAMP-dependent protein kinase. Phosphorylation of the myosin kinase results in a 20-fold decrease in the affinity of the kinase for calmodulin and a 1 1/2-fold decrease in the maximal rate (Vmax) of kinase activity. Dephosphorylation of phosphorylated myosin light chain kinase with a phosphatase isolated from smooth muscle restores the ability of the kinase to bind Ca^{2+} at the same level as the unphosphorylated enzyme.

Phosphorylation of myosin light chain kinase provides a mechanism by which stimulation of beta-receptors may result in smooth muscle relaxation. Interaction of epinephrine with the beta-receptor results in a rise in adenalate cyclase and hence increased levels of cAMP. The latter compound activates protein kinase to phosphorylate myosin kinase, resulting in a decrease in the activity of this enzyme. Decreased activity of myosin kinase favors the nonphosphorylated form of myosin, which is not capable of being activated by actin, and relaxation of smooth muscle actomyosin ensues.

The enzyme myosin light chain phosphatase, which is capable of dephosphorylating the 20,000 dalton myosin light chains, has been partially purified from turkey gizzards. Two separate fractions of phosphatase activity were eluted from Sepharose 4B. One fraction catalyzed dephosphorylation of both phosphorylated histones and phosphorylated myosin light chains and the second dephosphorylated myosin light chains, but not histone. This latter fraction is also capable of dephosphorylating the enzyme myosin light chain kinase.

Cardiac myosin light chain kinase The kinase catalyzing phosphorylation of cardiac myosin was isolated from canine ventricles. The kinase was purified to homogeneity as determined by electrophoresis in Na-dodecylsulfate-polyacrylamide gels and polyacrylamide gels run in the absence of a denaturing agent. The kinase has a molecular weight of 70,000, requires both Ca^{2+} and calmodulin for activity and has a specific activity of 1.2 umols P_i/mg/enzyme/min. The kinase can be phosphorylated by the catalytic subunit of cAMP-dependent protein kinase

Brain kinase and platelet myosin kinase and protease The kinase catalyzing the phosphorylation of the 20,000 dalton light chain of myosin was partially purified from bovine brain. A cAMP dependent protein kinase copurified with the myosin kinase through calmodulin affinity chromatography. The two kinases could be separated by glycerol gradient centrifugation.

The kinase catalyzing phosphorylation of platelet myosin light chains was purified from human platelets. Depending on the method of purification the kinase could be isolated in a Ca^{2+}-calmodulin dependent and a Ca^{2+}-calmodulin independent form. The latter appears to be a proteolytic fragment of the former kinase, which has a molecular weight of 105,000. The platelet kinase could be phosphorylated by the catalytic subunit of cAMP-dependent protein kinase. Phosphorylation of the platelet kinase markedly decreased the apparent affinity of the kinase for calmodulin

A Ca^{2+}-dependent protease, capable of degrading platelet myosin heavy chain (200,000 dalton) and light chain (20,000 dalton) as well as the 20,000 dalton light chain of smooth muscle myosin and the enzyme smooth muscle myosin kinase was isolated from human platelets. The protease has a molecular weight of approximately 84,000 daltons in SDS-polyacrylamide gel electrophoresis and is inactive in the presence of EGTA and the proteolytic inhibitor leupeptin. It may play an important role in the turnover of platelet myosin as well as the enzymes controlling platelet actin-myosin interaction.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01661-04 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
The Natural History of 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
	Jeffrey S. Borer	Senior Investigator	CB	NHLBI
	Lewis C. Lipson	Senior Investigator	CB	NHLBI
	Robert Bonow	Senior Investigator	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	.4	.1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

To determine the natural history of patients with coronary artery disease who have no or mild symptoms, 95 patients have been followed for an average of 2 years. The annual mortality is 1.5% per year and is considerably better than older natural history studies.

PHS-6040
(Rev. 10-76)

Project Description:
The role of coronary revascularization in the treatment of patients with coronary artery disease who are asymptomatic or mildly symptomatic on medical management is contingent on whether or not the operation prolonges life. Previous studies comparing medical vs operative treatment are based on medically treated patients with severe symptoms studied in the 1960s and early 1970s. Annual mortality of such patients averaged 7% and 11% in patients having two or three vessel disease, respectively. In a prospective study started 4-1/2 years ago at the N.I.H., patients who either had no symptoms (18%) or mild symptoms on propranolol and nitrates were admitted for coronary angiography. Eleven patients with left main coronary occlusion (<50% reduction in diameter) were excluded from the study and referred for operation. Ninety-five other patients have been followed from 6 to 45 months (average 2 years) to determine their clinical course. Approximately one-third of the patients had single vessel disease, one-third had double vessel disease and one-third had triple vessel disease. Left ventricular function was normal in 65% of the patients (ejection fraction >55%). All patients were content with their lifestyles and angina pectoris, if present, was mild and did not limit their day to day activity.

Three patients have died during follow-up period, two suddenly and unexpectedly. The third patient sustained an acute myocardial infarction, had progressive symptoms after the infarction and died while waiting for operation. Thus, the annual mortality (actuarial analysis) of the group as a whole was 1.5% per year and 2% per year for patients with double or triple vessel disease. An additional 8 patients experienced symptomatic progression 6 to 40 months after the initial evaluation (average 28 months), and subsequently underwent successful coronary revascularization. Thus, despite extensive coronary artery disease in these patients with no or mild symptoms, sudden death was infrequent and successful revascularization could be instituted in most patients after moderate to severe symptoms developed.

The results of these studies are quite different from previously reported natural history studies. This study indicates that the natural history of asymptomatic patients with coronary artery disease followed in the 1970s is considerably better than that suggested by previous studies of patients with more severe symptoms studied in the 1960s. Whether or not the differences are due to better medical management, the high incidence of normal left ventricular function, or the fewer symptoms in the patients is impossible to determine from this study.

These data suggest that a reasonable approach to patients without left main coronary artery disease who have few or no symptoms would be to follow such patients until symptoms progress to the point at which coronary revascularization can be used to improve the patient's symptomatology. The incidence of sudden death or the development of severe irreversible left ventricular dysfunction in asymptomatic or mildly symptomatic patients with coronary artery disease is not very high and patients who develop more severe symptoms can be operated upon successfully.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01665-04 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Phosphorylation and Regulation of Smooth Muscle Myosin

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

PI:	Mary Anne Conti	Graduate Student	CB	NHLBI
Other:	Robert S. Adelstein	Head, Molecular Cardiology	CB	NHLBI
	Claude B. Klee	Biochemist	LB	NCI
	C. Robert Eaton	Physicist, Molecular Cardiology	CB	NHLBI
	William Anderson, Jr.	Chemist, Molecular Cardiology	CB	NHLBI
	James Maurice Miles	Med. Biology Technician	CB	NHLBI

COOPERATING UNITS (if any)
Laboratory of Biochemistry, NCI, NIH.

LAB/BRANCH
Cardiology Branch

SECTION
Molecular Cardiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.8	1.2	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)

Myosin light-chain kinase can be phosphorylated by the catalytic subunit of cyclic AMP-dependent protein kinase. Phosphorylation of myosin kinase decreases the affinity of this enzyme for the calcium binding protein calmodulin by twenty fold. The ability of cyclic AMP dependent protein kinase to decrease the activity of myosin light chain kinase, provides a direct mechanism for cyclic AMP mediated smooth muscle relaxation.

PHS-6040
(Rev. 10-76)

Project Description: Phosphorylation of the 20,000 dalton light chain of smooth muscle myosin by the specific enzyme, myosin light chain kinase, is required for actin-activation of myosin Mg-ATPase activity. The purified kinase (130,000 daltons), which shows a single band in SDS and urea polyacrylamide gel electrophoresis has a specific activity of 30μmol PO_4 transferred to the light chain/mg kinase/min at 24°. Enzymmatic activity is independent of cAMP but is completely dependent on trace Ca^{2+}(10^{-8}M) and on the presence of the ubiquitous calcium-binding protein, calmodulin (CDR).

When myosin light chain kinase is incubated with the catalytic subunit of cAMP-dependent protein kinase, up to one mole of phosphate (from Mg-ATP) can be covalently incorporated per mole of myosin kinase. The rate of phosphorylation of myosin kinase is comparable to the rate of phosphorylation of histone by the cAMP-dependent protein kinase. Brief tryptic digestion of ^{32}P-labelled kinase (1:250 mg trypsin:kinase, 0°, 1-4 min) results in the release of a single radioactively-labelled tryptic peptide of molecular weight 23,000, suggesting that a single site in the myosin kinase is being phosphorylated. Concommitant with the release of the 23,000 dalton peptide there is the appearance of an 80,000 to 100,000 dalton peptide, indicating that the tryptic peptide is released from either the carboxyl- or amino-terminus of the kinase.

Phosphorylation of myosin light chain kinase decreases the rate at which the kinase phosphorylates myosin light chains by lowering the affinity of the kinase for its activator, calmodulin. The phosphorylated kinase has a 20-fold weaker binding constant for calmodulin than the unphosphorylated kinase (K_a2.5x 10^{-8} and 1.2x 10^{-9}M respectively). In contrast, phosphorylation of the kinase decreases by 50% the maximal rate (V_{max}) of enzymmatic activity.

Phosphorylation of myosin kinase by cAMP-dependent protein kinase suggests a mechanism for the direct effect of cAMP on actin-myosin interaction in smooth muscle. Experiments are in progress to investigate the structural and functional relation between the phosphorylated site and the calmodulin-binding site on the myosin kinase. Tryptic digestion will be used to cleave the kinase followed by calmodulin-affinity chromatography and gel filtration to separate and characterize the peptides produced. Peptides will also be assayed for their enzymmatic activity, ^{32}P content and for their ability to inhibit the activity of native myosin kinase by binding to calmodulin.

Publications:

1) Adelstein, R.S., Conti, M.A., Hathaway, D.R., and Klee, C.B.: Phosphorylation of smooth muscle myosin light chain kinase by the catalytic subunit of adenosine 3':5'-monophosphate dependent protein kinase. J Biol Chem 253: 8347-8350, 1978.

2) Trotter, J.A. and Adelstein, R.S.: Macrophage Myosin : Regulation of Actin-activated ATPase activity by phosphorylation of the 20,000 dalton light chain. J Biol Chem, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01670-03 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

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:	Jeffrey S. Borer	Senior Investigator	CB	NHLBI
	Kenneth M. Kent	Head,Cardiovascular Diagnosis	CB	NHLBI
	Stuart F. Seides	Senior Investigator	CB	NHLBI
	Robert O. Bonow	Senior Investigator	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.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)

Sixty-two 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 better 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. 10-76)

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, 62 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 14 months. This group has been divided into 15 asymptomatic patients with mild aortic regurgitation, 41 asymptomatic patients with moderate to severe AR, and 6 patients who have undergone aortic valve replacement after having been initially followed in the Natural History Study. These latter six were operated on either because of decreasing left ventricular function or the development of symptoms while under observation. No patient from this group has died at this point.

In comparison to this group, the same intensive evaluation has been carried out on 35 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 five deaths in this group. Three 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 from the present study, but also from data obtained on patients evaluated prior to initiation of this study. Valve replacement is performed not only with patients with significant symptomatology, but also when left ventricular systolic function is compromised as evidenced by echocardiography (left ventricular systolic dimension greater than 55mm).

Exercise radionuclide angiography and exercise-induced changes in pulmonary capillary wedge-pressure also are measurements capable of distinguishing the three groups of mildly or asymptomatic patients listed above. However, since only six 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.

This study is being continued, with the aim of developing further predictive indices that will be sensitive enough to permit operation to be performed before the development of irreversible left ventricular failure.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01672-03 CB

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Establishment 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:	Roger H. Dailey	Computer System Analyst	DMB	DCRT
	Gerald D. Stoner	Head,Applied Syst. Prog. Sec.	DMB	DCRT
	Gail J. Greenberg	Computer Technician	CB	NHLBI
	Kenneth M. Kent	Head, Cardiovascular Diagnosis	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

Div. of Computer Research and Technology, NIH (Data Management Branch)

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, and radionuclide angiogram results.

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, 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 will be generated for the medical records. Data accumulation was begun on January 1, 1978 and current and retroactive information has been entered on over 90% 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 will be merged into one system, thus facilitating access to data on all patients with cardiovascular disease. Frequent querying of the data base for Clinical and research purposes has occurred. Consideration is being given to producing a summary of available"pertinent" clinical data for each outpatient visit in order to make chart reviews easier at these visits.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01684-03 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Myocardial Infarction in Hypertrophic Cardiomyopathy

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

PI:	Barry J. Maron	Senior Investigator	CB NHLBI
Other:	William C. Roberts	Chief, Pathology Branch	PB NHLBI
	Dennis E. Chenowith	Resident in Pathology	Univ.of Cal.,at Irvine
	Stephen E. Epstein	Chief, Cardiology Branch	CB NHLBI

COOPERATING UNITS (if any)

Department of Pathology - University of California at Irvine
Pathology Branch, NHLBI

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.1	.06	.04

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Transmural myocardial infarction was identified in six patients with hypertrophic cardiomyopathy and normal coronary arteries. Transmural myocardial infarct presented as sudden death, classical infarct or was silent. While left ventricular dilatation is rare in hypertrophic cardiomyopathy, one of the factors predisposing to this unusual feature appears to be transmural myocardial infarction.

PHS-6040
(Rev. 10-76)

Project Description: Patients with hypertrophic cardiomyopathy often have angina pectoris with ECGs showing evidence of myocardial necrosis or ischemia. Such patients may actually have coronary artery disease which may result in acute transmural myocardial infarction. However, the fact that patients with hypertrophic cardiomyopathy may also have transmural myocardial infarction with normal coronary arteries has not been appreciated. Among 45 patients with hypertrophic cardiomyopathy studied at necropsy, 8 patients (aged 12 to 60 years) had transmural myocardial infarction primarily involving the ventricular septum, without significant (>75% reduction in cross-sectional area) coronary arterial narrowing by atherosclerosis. In 5 of the 8 patients clinical manifestations of transmural myocardial infarction were absent, although 2 patients had cardiac arrests that probably were due to transmural myocardial infarction; in 1 patient transmural myocardial infarction presented in the typical clinical fashion. Of note, dilatation of both ventricular cavities, an unusual finding in hypertrophic cardiomyopathy, was present in 7 of the 8 patients. Thus, transmural myocardial infarction (in the absence of coronary arterial disease) may influence the natural history of patients with hypertrophic cardiomyopathy. Clinically, in patients with hypertrophic cardiomyopathy, transmural myocardial infarction may present as sudden death, classical transmural myocardial infarction or may be "silent". Finally, while ventricular dilatation is infrequent in hypertrophic cardiomyopathy, one of the factors predisposing to this unusual feature is transmural myocardial infarction.

Publications: Maron, B.J., Roberts, W.C., Chenowith, D.E., and Epstein, S.E.: Am J Cardiol 43: 1086-1102, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01689-03 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Phosphorylation of Cardiac Myosin

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

PI:	Lydie Rappaport	Expert	CB	NHLBI
Other:	Robert S. Adelstein	Head, Molecular Cardiology	CB	NHLBI
	William Anderson, Jr.	Chemist	CB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cardiology Branch

SECTION
Molecular Cardiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.4	1.2	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)

Cardiac myosin light chain kinase was purified from strips of ventricle and found to have a molecular weight of 70,000 in SDS-polyacrylamide gel electrophoresis. The enzyme requires both Ca^{2+} and calmodulin for activity and has a specific activity of 1.2 μmoles P_i/mg kinase/min. Cardiac myosin kinase can be phosphorylated by the catalytic subunit of cAMP-dependent protein kinase, as determined by radioautography.

PHS-6040
(Rev. 10-76)

Project Description: The enzyme catalyzing phorphorylation of the 20,000 dalton light chain of myosin was purified from strips of canine cardiac ventricles using calmodulin affinity chromatography. The kinase was purified to homogeneity as determined by both SDS-polyacrylamide gel electrophoresis and gel electrophoresis at pH 8.0 under non-denaturing conditions. The enzyme has a molecular weight of 70,000 in SDS-PAGE and a specific activity of 1.2 μmoles P_i transferred to myosin light cahin/mg kinase/min. The catalytic subunit of cAMP-dependent protein kinase phosphorylated the 70,000 dalton myosin light chain kinase, as determined by radioautography. Present studies are directed toward quantitating the extent of myosin light chain kinase phosphorylation and determining what effect, if any, phosphorylation of the myosin kinase has on activity.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01700-03 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Changes in Platelet Survival and Heart Rate Responses After Propranolol.

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

PI:	Robert Goldstein	Senior Investigator	CB	NHLBI
Other:	Lawrence Corash	Asst.Chief,Hematology Service	PB	CC
	John Tallman, Jr.	Actg.Chief,Section of Biologics	BPB	NIMH
	C. Raymond Lake	Staff Psychiatrist	LCS	NIMH
	John Hyde	Math.Medical Statistitian	MSAB	HL
	Judith Anderson	Physician	PB	CC
	Stephen Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

National Institute of Mental Health, NIH.
Pathology Branch, CC, NIH.

LAB/BRANCH
Cardiology Branch

SECTION
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.3	.25	.05

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Physiologic responses to abrupt withdrawal of propranolol were examined in 14 normal subjects. Heart rate during exercise and upright tilt was slightly increased after propranolol when compared with prepropranolol performance. Platelet survival was shortened 22% after propranolol. However, heart rate response to isoproterenol, beta receptor function of white blood cells, and levels plasma catecholamine were unchanged during propranolol withdrawal. Increased platelet deposition in coronary stenoses may play a role in ischemic sequelae following abrupt propranolol withdrawal.

Project Description: Although abrupt discontinuation of propranolol (P) may precipitate myocardial ischemia dn infarction in coronary patients, physiologic consequences of abrupt withdrawal (W) of P are not fully understood. Therefore we examined platelet survival and heart rate (HR) responses to exercise, upright tilt, and isoproterenol (I) in 14 normal subjects before P and after abrupt W of P, 80-240 mg/d given 24-79 days. P effect was confirmed by lower HR during exercise and I infusion. In 10 subjects mean survival time of ^{51}Cr tagged platelets fell from 10.0 days before P to 7.8 days after W ($p<.05$). One day after W HR rise with tilt was increased, but HR responses were otherwise unchanged from pre-P. Two days after W, however, mean peak HR during exercise (165 bpm) was 12 bpm higher ($p<.01$) than pre-P. On this same day HR rose more after unmedicated tilt (+7 bpm, p .05) and more after tilt following vagal blockade (+7 bpm, p .02) when compared with pre-P values. Eight days after W, HR responses to exercise or tile remained increased. HR responses to I infusion (5-40 ng/kg/min, n=14), white blood cell (WBC) β-receptor function (cyclic AMP production after I and dihydroalprenolol binding, n=9) and plasma norepinephrine at rest and during exercise (n=7) were each unaltered during W. Our results suggest that abrupt W of P is accompanied by increase in platelet utilization and enhancement of sympathetically-mediated reflex rises in HR. These changes may each play a role in the increased incidence of ischemic episodes observed after W of P from coronary patients. However, the number of β-receptors (per WBC data) and their sensitivity to adrenergic agonist did not seem changed by abrupt W of P.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01703-03 CB

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Ventricular Function During Exercise Before and After Aortic Valve Replacement

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

PI:	J.S. Borer	Senior Investigator	CB	NHLBI
Other:	S.L. Bacharach	Physicist	NM	CC
	M.V. Green	Chief, Applied Physics Sec.	NM	CC
	K.M. Kent	Head, Cardiovasc. Diagnosis	CB	NHLBI
	D.R. Rosing	Senior Investigator	CB	NHLBI
	S.R. Seides	Senior Investigator	CB	NHLBI
	C.L. McIntosh	Senior Surgeon	SB	NHLBI
	D.M. Conkle	Senior Surgeon	SB	NHLBI
	A.G. Morrow	Chief, Surgery Branch	SB	NHLBI
	S.E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

Surgery Branch, NHLBI
Nuclear Medicine Department, CC, NIH

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.3	.2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Radionuclide cineangiography during exercise allows accurate characterization of the functional reserve to the left ventricle. We have used the technique routinely to evaluate the status of patients with aortic regurgitation after aortic valve replacement, and have compared these results with the findings in the same patients before operation. While LV function during exercise improves markedly after operation, it does not return to normal.

PHS-6040
(Rev. 10-76)

Project Description: To determine the effect of aortic valve replacement on reversing abnormalities of left ventricular function in patients with aortic regurgitation, we used radionuclide cineangiography to study 16 symptomatic patients with aortic regurgitation prior to and six months after aortic valve replacement. Prior to operation, left ventricular ejection fraction was 46 ± 3% at rest (normal = 57 ± 1%, $p < .001$), and fell to 37 ± 4% during exercise (normal = 71 ± 2%, $p < .001$). After operation, ejection fraction at rest rose to 58 ± 4%, indistinguishable from normal, and ejection fraction during exercise was 53 ± 4%, increased ($p < .001$) from prior to operation, but significantly ($p < .001$) subnormal. Thus, aortic valve replacement can improve, but usually does not normalize, left ventricular function during exercise in symptomatic patients with aortic regurgitation.

Publications: Amer. J. Cardiol. (Dec. 1979), in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01704-03 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Comparison of Radionuclide Cineangiography and Exercise Electrocardiography

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

PI:	J.S. Borer	Senior Investigator	CB	NHLBI
Other:	K.M. Kent	Head, Sec. on Cardiovasc. Diagnosis	CB	NHLBI
	S.L. Bacharach	Physicist	NM	CC
	M.V. Green	Chief, Applied Physics Sec.	NM	CC
	S.E. Epstein	Chief, Cardiology Branch	CB	NHLBI
	G.S. Johnston	Chief, Nuclear Medicine Dept.	NM	CC

COOPERATING UNITS (if any)

Dept. of Nuclear Medicine, CC, NIH.

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Betehsda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	.4	.1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Radionuclide cineangiography is a sensitive method to detect ischemic heart disease due to coronary artery stenosis. We have compared the sensitivity of the test with that of a more commonly used procedure, exercise electrocardiography, and have found the radionuclide procedure significantly more sensitive.

Project Description: Non-invasive radionuclide cineangiography permits assessment of global and regional left ventricular function during intense exercise. To assess the sensitivity of the technique in detecting coronary artery disease, we studied 63 consecutive patients with ≥ 50 percent stenosis of at least one coronary artery. Fifty-nine (94 percent) demonstrated regional dysfunction with exercise; 56 (89 percent) developed ejection fractions during exercise below the limits of normal. When both regional dysfunction and subnormal ejection fractions are considered together, sensitivity was 95 percent. Each patient also underwent exercise electrocardiography to either angina or 85 percent predicted maximal heart rate. Of the 42 patients who developed angina during exercise electrocardiography, 26 (62 percent) developed ≥ 1 mm ST segment depression; four additional patients (10 percent) manifested Q waves diagnostic of previous myocardial infarction. In contrast, 39 (93 percent, $p < .001$) developed regional dysfunction during radionuclide study, and one additional patient developed a subnormal ejection fraction without regional dysfunction. To assess specificity we studied 21 consecutive patients with chest pain but normal coronary arteries. None developed regional dysfunction; ejection fraction increased in all to levels within the range previously defined as normal. Predictive accuracy in this symptomatic population was 100%. We concluded that radionuclide cineangiography is highly sensitive (more so than exercise electrocardiography), predictive and specific in the detection of patients with coronary artery disease.

Publications:

Borer, J.S., Kent, K.M., Bacharach, S.L., Green, M.V., Epstein, S.E., and Johnston, G.S.: Comparison of Radionuclide Cineangiography and Exercise Electrocardiography. Circulation, Sept. 1979 (In press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01705-03 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Conduction System Abnormalities in Patients with ASH

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

PI:	J.S. Borer	Senior Investigator	CB NHLBI
Other:	R. Fletcher	Chief, Cardiology Div.	Washington VA Hosp.
	A. Del Negro	Head, Cath. Lab.	Washington VA Hosp.
	S.F. Seides	Senior Investigator	CB NHLBI
	D.R. Rosing	Senior Investigator	CB NHLBI
	K.M. Kent	Head, Sec. Cardiovasc. Diagnosis	CB NHLBI
	S.E. Epstein	Chief, Cardiology Branch	CB NHLBI

COOPERATING UNITS (if any)
Cardiology Division - Washington VA Hospital

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.1	.08	.02

CHECK 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 ASH, with or without obstruction, have an alarming proclivity for the development of syncope or sudden death. Since this may be due to abnormalities in the cardiac conduction system, we have used His Bundle Electrography to study 27 patients with syncope or family history of sudden death. We have found several conduction system abnormalities in patients in this group which might be related to sudden death.

PHS-6040
(Rev. 10-76)

Project Description: In some families with hypertrophic cardiomyopathy (HCM), sudden death occurs with high frequency, particularly in family members with very thick ventricular septae. Other investigators recently reported electrophysiological abnormalities (EA) in >90% of pts with obstructive HCM and syncope. To elucidate EA potentially predictive of sudden death, we studied 36 pts with HCM: 18 pts (from 13 different families) had ≥2 first degree relatives who had died suddenly of HCM ("malignant family history" (MFH)). The remaining 18 pts manifested syncope (11 pts), palpitations (9 pts), dyspnea (11 pts) and/or chest pain (12 pts), but did not have MFH. Four of the 36 pts had suffered spontaneous ventricular fibrillation (VF). At least one major EA was present in 20 of the 36 pts: most notably, 18 manifested prolonged H-V times (>55 msec, range 60 to 95 msec): 2 pts had accessory A-V conduction pathways. These abnormalities were found in 2 of the 4 pts who experienced spontaneous VF but did not correlate with MFH or presence or absence of LV outflow gradient. However, septal thickness, while unrelated to MFH, was closely related to H-V prolongation. The septum was ≥25 mm in 13 of 18 pts with H-V >55 msec and in only 3 of 18 with normal H-V ($p<.001$). We conclude that EA, particularly prolonged H-V, are frequent in pts with HCM, irrespective of outflow obstruction. The strong correlation of EA with septal thickness, a known correlate of sudden death in pts with HCM, suggests that EA may be involved in the mechanism of death in these pts. Further follow-up will determine whether EA can predict sudden death in HCM.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01706-03 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Prognostic Value of Non-invasive Assessment after Acute Myocardial Infarction

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

PI:	Jeffrey S. Borer	Senior Investigator	CB NHLBI
Other:	Kenneth M. Kent	Head, Cardiovascular Diagnosis	CB NHLBI
	Douglas R. Rosing	Senior Investigator	CB NHLBI
	Stuart F. Seides	Senior Investigator	CB NHLBI
	Stephen L. Bacharach	Physicist	NM CC
	Michael V. Green	Chief,Applied Physics Sec.	NM CC
	David Holmes	Physician	Nat. Naval Med. Center
	Howard Cohen	Physician	" " " "
	William Baker	Physician	" " " "
	Dennis Donohue	Physician	Walter Reed Army Med.Ctr
	Jules Bedynek	Physician	" " " " "
	Stephen E. Epstein	Chief, Cardiology Branch	CB NHLBI

COOPERATING UNITS (if any)
Nuclear Medicine Dept. - CC - NIH
National Naval Medical Center - Bethesda, Md.
Walter Reed Army Medical Center - Washington, D.C.

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.3	.2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Assessment of left ventricular function at rest and analysis of arrhythmias prior to hospital discharge allows prediction of a portion of the 10-15% of patients who will die suddenly within six months of discharge after acute myocardial infarction. Using new non-invasive radionuclide cineangiographic techniques, which permit study during exercise and hence provide more complete assessment of cardiac function than previously available, we found that prediction of death within a year after infarction was not improved by assessment during exercise. However, though no significant change in left ventricular function occurred in the group during the course of the year after infarction, assessment of function during exercise did permit selection of a subgroup of patients manifesting significant functional improvement.

PHS-6040
(Rev. 10-76)

Project Description: To clarify the natural history of left ventricular function after acute myocardial infarction we studied 45 patients (40 in Killip Classes I ± II) one day prior to hospital discharge (2-4 weeks after infarction) and again 6 to 13 months later. Initial study included: 1) 24-hour ambulatory ECG monitoring and 2) Tc^{99m} radionuclide cineangiography, electrocardiography and plasma norepinephrine assay, each performed at rest and during submaximal exercise (to angina or heart rate to 115). Radionuclide cineangiography, ECG and norepinephrine assays were repeated at rest, submaximal and maximal exercise at the later study. Ejection fraction (EF) at rest generally was subnormal at the early study, ranging from 10% to 59% (average $39\pm5\%$, normal average $57\pm1\%$, $p<.001$), and showed little change during submaximal exercise (average $37\pm5\%$, normal average $67\pm1\%$, $p<.001$). EF correlated significantly with the frequency and complexity of ventricular arrhythmias during 24-hour electrocardiography, but neigher exercise ECG ST segments nor plasma norepinephrine content correlated with EF or with arrhythmia frequency or complexity. Four of the 45 patients died within one year of infarction; each had EF <35% at rest at the early study, but neigher presence of complex arrhythmias nor determination of EF during exercise provided additional help in predicting those who died. Among the 41 survivors, EF at the late study (average $42\pm4\%$ at rest and $40\pm4\%$ during submaximal exercise) was not significantly different from EF at the early study. However, the subgroup of 17 patients with exercise EF >40% at the early study showed a small but significant increase in EF at the later study. Those with exercise EF $\leq$40% at the early study showed no change at later study. During the late study, EF at maximal exercise ($39\pm4\%$) was indistinguishable from EF at submaximal exercise and was not significantly different than EF at rest. This finding was markedly different from that in our patients with coronary disease but wihtout infarction, or patients with remote (>3 years pre-study) infarction, who as a group have manifested marked (<.001) fall in EF with exercise. The absence of significant reduction in EF from rest to exercise during the year after infarction suggests that relatively little potentially ischemic tissue remains early after infarction; progression of coronary artery obstruction during ensuing years probably accounts for the more typical response of EF to exercise, and for subsequent coronary events.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01709-03 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Effect of Propranolol During Exercise in Coronary Artery Disease

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

PI:	J.S. Borer	Senior Investigator	CB	NHLBI
Other:	S.L. Bacharach	Physicist	NM	CC
	M.V. Green	Chief, Applied Physics Sec.	NM	CC
	K.M. Kent	Head, Cardiovascular Diagnosis	CB	NHLBI
	D.R. Rosing	Senior Investigator	CB	NHLBI
	S.F. Seides	Senior Investigator	CB	NHLBI
	S.E. Epstein	Chief, Cardiology Branch	CB	NHLBI
	R.O. Bonow	Clinical Associate	CB	NHLBI

COOPERATING UNITS (if any)
Nuclear Medicine Department, CC

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.1	.08	.02

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Beta adrenergic blockade with propranolol can relieve angina pectoris, and may improve survival in some patients with coronary artery disease. Because it reduces contractility, it may cause clinically important left ventricular dysfunction in some patients with coronary artery disease. Therefore propranolol is often withheld if evidence of left ventricular dysfunction exists in the absence of drug. However, it is possible that by reducing myocardial oxygen demand in such patients, propranolol might actually prevent ischemic dysfunction, which otherwise might occur during stress. We tested the efficacy of propranolol in this setting with the use of non-invasive radionuclide cineangiography, and determined that the drug cuases mild depression of function in non-ischemic regions of the left ventricle during exercise, and spares the function of ischemic regions during exercise.

PHS-6040
(Rev. 10-76)

Project Description: Propranolol reduces ejection fraction at rest and during exercise in normal subjects. Its effect on left ventricular function in patients with coronary artery disease is unclear. At rest, contrast angiography indicates that propranolol impairs left ventricular function; radionuclide studies have shown no effect. To resolve this controversy we used radionuclide cineangiography at rest and during angina-limited exercise to study 20 patients before and after propranolol (.05-.1 mg/kg iv.). Exercise protocols were identical before and after propranolol. At rest, propranolol caused an average 11 beat/min fall in heart rate. No new wall motion abnormalities developed, though ejection fraction fell slightly (ejection fraction before propranolol = 57%, after propranolol = 51%, $p<.005$). After propranolol, only 8 of 20 developed angina with exercise; heart rate was reduced 12 beats/min at peak exercise ($p<.001$). Ejection fraction during exercise before propranolol was 45%, after propranolol 42% ($p<.02$); no new regional dysfunction was noted after propranolol. After propranolol, depression of ejection fraction was significantly less ($p<.05$) during exercise than at rest. While regional ejection fraction during exercise was slightly depressed (-4.5%) in regions which were normal during exercise before propranolol, there was a very small (+1.5%) increase in ejection fraction in regions of the left ventricle which were ischemic during exercise before propranolol. (Difference in response of the two types of regions was significant, $p<.05$). Thus, at effective anti-anginal levels, propranolol causes mild depression of global left ventricular function at rest, when little ischemia is present. During ischemia-producing exercise, only minimal global depression is noted, even in ischemic regions, however, may be spared or even improved during exercise after propranolol.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01712-02 CB

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Ca^{2+}-dependent Enzymes from Human Platelets and Bovine Brain

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

PI:	David R. Hathaway	Clinical Associate	CB	NHLBI
Other:	Robert S. Adelstein	Head, Molecular Cardiology	CB	NHLBI
	William Anderson, Jr.	Chemist	CB	NHLBI
	James Maurice Miles	Med. Biology Technician	CB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Molecular Cardiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.2	0.4

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The kinases catalyzing the phosphorylation of the 20,000 dalton light chain of myosin has been isolated from bovine brain and human platelets. The platelet kinase has been isolated in a Ca^{2+}-calmodulin dependent and independent form. A Ca^{2+}-dependent protease capable of degrading certain contractile proteins from platelets and smooth muscles has also been isolated from human platelets.

PHS-6040
(Rev. 10-76)

Project Description:

Brain myosin light chain kinase: The kinase catalyzing phosphorylation of the 20,000 dalton light chain of myosin was partially purified from bovine brain. A cAMP-dependent protein kinase co-purified with the myosin kinase through the calmodulin affinity chromatography step. The two kinases could be separated by sedimentation in a glycerol gradient. The kinase catalyzing phosphorylation of the myosin light chain was eluted from a non-denaturation gel and the peak of activity was found to correlate with a protein band of 130,000-140,000 daltons following SDS-polyacrylamide gel electrophoresis. This would imply that the myosin kinase isolated from brain is significantly larger than that isolated from platelets.

Platelet myosin light chain kinase: The kinase catalyzing phosphorylation of the 20,000 dalton light chains of platelet myosin was purified from human platelets. Depending on the method of purification the kinase could be isolated in a Ca^{2+}-calmodulin dependent and a Ca^{2+}-calmodulin independent form. The latter appears to be a proteolytic fragment of the former kinase, which has a molecular weight of 105,000 daltons. Unlike the Ca^{2+}-calmodulin dependent kinase the independent kinase was incapable of binding to a calmodulin-Sepharose 4B affinity column in the presence of Ca^{2+}. The Ca^{2+}-calmodulin dependent kinase could be phosphorylated by the catalytic subunit of cAMP-dependent protein kinase. Phosphorylation of the platelet kinase markedly decreased the apparent affinity of the kinase for calmodulin.

Platelet protease: A Ca^{2+}-dependent protease, capable of degrading the following proteins was isolated from human platelets: 1) platelet myosin heavy chain (but not smooth muscle myosin heavy chain), 2) the 20,000 dalton light chain of platelet and smooth muscle myosin, 3) smooth muscle myosin light chain kinase. The platelet protease has a molecular weight of 84,000 daltons in SDS-polyacrylamide gel electrophoresis and is inactive in the presence of EGTA, the proteolytic inhibitor leupeptin, and following modification of its sulfhydryl groups. This protease may play an important role in the turnover of platelet myosin as well as the enzymes regulating platelet actin-myosin interaction.

Publications:

Adelstein, R.S., Conti, M.A., and Barylko, B: The role of myosin phosphorylation in regulating actin-myosin interaction in human blood platelets. Thrombosis and Halmostosis (Stuttg) 40: 241-244, 1978.

Hathaway, D.R. and Adelstein, R.S.: Human platelet myosin light-chain kinase requires the calcium-binding protein calmodulin for activity. Proc Natl Acad Sci USA 76: 1653-1657, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01715-02 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Myocardial Infarct Size as a Function of Myocardium at Risk: Influence of Aspirin

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:	L.C. Lipson	Senior Investigator	CB	NHLBI
	F.H. Sheehan	Clinical Associate	CB	NHLBI
	N.L. Capurro	Senior Staff Fellow	CB	NHLBI
	J.M. Isner	Clinical Associate	CB	NHLBI
	R.E. Goldstein	Senior Investigator	CB	NHLBI
	W.C. Roberts	Chief, Pathology Branch	PB	NHLBI
	S.E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

Pathology Branch, NHLBI

LAB/BRANCH
Cardiology Branch

SECTION
Section on Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	.4	.1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Previous studies have demonstrated that pretreatment with platelet inhibitory doses of aspirin (ASA) augments epicardial collateral flow by >50% four hours after ligation of the left anterior descending coronary artery (LAD) in dogs. To determine whether this effect is suffucient to reduce the size of subsequent myocardial infarction, we administered ASA to 17 dogs and saline to 16 control dogs prior to LAD occlusion. After 3 days, animals were sacrificed. In vitro perfusion of the aortic root with Evan's blue and of the LAD distal to occlusion identified the unstained myocardium as the region at risk of infarction. Further staining of this region with tetrazolium identified viable from infarcted tissue. ASA treated dogs showed no difference from controls in %LV at risk, in % infarct/LV, and in % infarct/risk region. Thus, despite the potentially beneficial increase in epicardial collateral flow produced by ASA pre-treatment, we found that ASA failed to significantly reduce infarct size.

PHS-6040
(Rev. 10-76)

Project Description: Epidemiologic and clinical studies suggest that aspirin (ASA) and other platelet-inhibitory drugs may be beneficial in pts with coronary artery disease. The nature of such beneficial action, however, has not been fully explored. We showed previously that pretreatment with platelet-inhibitory doses of ASA (3 mg/kg) augments epicardial collateral flow by >50% ($p < .05$) 4 hrs after ligation of the left anterior descending coronary artery (LAD). No change occurred in endocardial flow. To determine whether this favorable influence of ASA is sufficient to decrease the amount of infarcted tissue, we administered either iv ASA, 3 mg/kg ($n = 17$), or saline ($n = 16$) to open-chest dogs 10 min prior to LAD occlusion. IV saline or ASA were repeated every 24 hrs. By 76 hrs 5 dogs died in each treatment group. Survivors were sacrificed at 76 hrs. The portion of the left ventricle (LV) at risk of infarction was delineated by aortic root perfusion with Evan's blue and simultaneous perfusion of the distal LAD with saline under equal physiologic pressures. Slices of the stained heart were incubated with tetrazolium to grossly identify infarct (with histologic confirmation). Total masses of LV, portion at risk, and infarct were measured for each dog. A direct relationship was found between mass at risk and mass infarcted ($r = 0.84$, $p < .001$). ASA-treated dogs showed no difference from controls in % LV at risk (34 ± 2 SE vs 36 ± 2), % infarct weight/LV weight (29 ± 3 vs 32 ± 2), and % infarct weight/weight of LV at risk (78 ± 4 vs 77 ± 3). Thus, despite ASA's ability to inhibit platelet aggregation and to increase epicardial collateral flow by >50%, we found that ASA treatment failed to reduce infarct size in this dog model of acute myocardial infarction.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01717-02 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Graded Exercise Testing in Symptomatic Patients with Aortic Regurgitation

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

PI:	Robert O. Bonow	Senior Investigator	CB NHLBI
Other:	Walter L. Henry	Senior Investigator	CB NHLBI
	Alan S. Pearlman	Senior Investigator	CB NHLBI
	Daniel D. Savage	Senior Staff Fellow	CB NHLBI
	Douglas R. Rosing	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
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	.4	.1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Graded treadmill exercise testing was performed in 52 symptomatic patients with aortic regurgitation (AR). Exercise capacity did not correlate with any hemodynamic or echocardiographic index of left ventricular (LV) function or size. However, among patients with echocardiographic evidence of LV systolic dysfunction (LV systolic dimension >55 mm or fractional shortening ≤25%), patients with good exercise capacity preoperatively had improvement in systolic function postoperatively, while patients with poor exercise capacity preoperatively had further deterioration of systolic function postoperatively. Postoperative survival was significantly greater ($p<.05$) in patients with LV systolic dysfunction with good exercise capacity compared to those with poor exercise capacity. Preoperative exercise testing is imprecise in assessing LV function in symptomatic patients with AR, but in combination with echo data is useful in predicting the outcome of subsequent operation and the reversibility of LV systolic dysfunction.

PHS-6040
(Rev. 10-75)

Project Description: To assess the role of graded treadmill exercise testing in managing patients with aortic regurgitation (AR), 52 symptomatic patients were evaluated preoperatively. Exercise performance was judged by exercise duration and maximum oxygen consumption (VO_2); patients unable to complete the 22.5 min of an exercise protocol were defined as having "poor" exercise capacity. Surprisingly: 1) patients with echo evidence of severe left ventricular (LV) systolic dysfunction (LV systolic dimension >55mm and fractional shortening <25% exercised as well as patients with normal LV function, and 2) patients with severe LV dilatation (LV diastolic dimension >75mm) exercised longer and to higher levels of VO_2 ($p<.01$) than those with milder degrees of dilatation. Exercise capacity did not correlate with LV end-diastolic pressure, pulmonary wedge pressure, or cardiac index obtained at catheterization. However, among patients with severe LV systolic dysfunction, whom we have previously shown to be at high risk postop of dying from congestive heart failure (CHF): 1) those with good exercise duration showed a decrease in LV systolic dimension postop (mean 64 to 41 mm, $p<.001$), fractional shortening increased (mean 24% to 32%, $p<.01$), and 0 of 7 have died; 2) those with poor exercise capacity showed no change in LV systolic dimension postop, fractional shortening deteriorated (mean 19% to 14%, $p<.05$), and 6 of 10 died from CHF. Postoperative survival was significantly greater ($p<.05$) in patients with LV systolic dysfunction with good exercise capacity compared to those with poor exercise capacity. Thus preoperative exercise testing is imprecise in assessing LV function in symptomatic patients with AR but in combination with echo data is useful in predicting the outcome of subsequent operation and the reversibility of LV systolic dysfunction.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01719-02 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

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 supraventricular tachyarrhythmias in two of two patients with hypertrophic cardiomyopathy. It has produced a significant reduction in premature ventricular beats in 2/5 patients, but has had no effect on premature supraventricular beats in two patients with this disorder. Verapamil has proved most beneficial in patients with atrial fibrillation.

PHS-6040
(Rev. 10-76)

Project Description: Verapamil, a calcium antagonist, 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 tachyarrhythmias may be a major mechanism for sudden death in this group. Because of its antiarrhythmic properties and the beneficial hemodynamic effects observed in patients with hypertrophic cardiomyopathy, verapamil has been administered to 15 patients with clinically significant arrhythmias, 10 supraventricular and five ventricular.

Eight patients had either paroxysmal or chronic atrial tachyarrhythmias. Oral verapamil was administered to five with chronic atrial fibrillation, and this drug was effective as a single agent in controlling the ventricular response to this arrhythmia. Four received excellent clinical benefit as a result of the rate control provided by verapamil, as well as its beneficial hemodynamic effects. One patient demonstrated good rate control, but still remained symptomatic and was switched to other medication. One patient with paroxysmal atrial fibrillation has now been free of this arrhythmia for three months while on verapamil. Two patients with paroxysmal atrial tachycardia as well as two patients with this arrhythmia who do not have hypertrophic cardiomyopathy showed marked symptomatic improvement through verapamil's ability to reduce or eliminate the frequency of the occurrence of this arrhythmia. Verapamil was of no benefit in suppressing premature supraventricular contractions in two patients with this arrhythmia.

Five patients with frequent premature ventricular contractions (greater than 30/hr were monitored on and off verapamil, and two showed a greater than 75% reduction in the frequency of the ventricular ectopic beats. In the other three patients, verapamil had no significant effect on the frequency of the VPBs.

In summary, verapamil seems to be a very effective antiarrhythmic agent for patients with 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 some patients.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01720-02 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Hemodynamic 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
Kenneth M. Kent	Head, Cardiovascular Diagnosis	CB	NHLBI
Stuart F. Seides	Senior Investigator	CB	NHLBI
Jeffrey S. Borer	Senior Investigator	CB	NHLBI
Barry J. Maron	Senior Investigator	CB	NHLBI
Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, 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)

Verapamil, a calcium antagonist, reduced left ventricular outflow obstruction in patients with hypertrophic cardiomyopathy, and produced no adverse effects on left ventricular function. These results suggest verapamil may be useful in the clinical management of patients with hypertrophic cardiomyopathy.

PHS-6040
(Rev. 10-76)

Project Description: Various investigators have proposed that abnormal Ca metabolism may contribute to the pathologic and physiologic abnormalities in some experimental cardiomyopathies. To help assess this possibility, the Ca antagonist verapamil was infused in 27 patients with hypertrophic cardiomyopathy (HCM) at the time of catheterization. Basal and provoked left ventricular outflow tract gradients were measured under control (C) conditions and during 3 progressively greater doses of verapamil (0.007, 0.014, 0.021 mg/kg/min). The peak verapamil effects on left ventricular outflow tract gradients in patients who had a gradient of at least 30 mmHg in the C state were: (mean ±SEM).

	Basal	Valsalva	Amyl NO_2	Isoproterenol
Control	84 ± 6	90 ± 8	82 ± 9	106 ± 14
Verapamil	56 ± 7†	64 ± 10†	43 ± 11*	63 ± 11*

Heart rate (HR), systolic blood pressure (SBP), mean pulmonary artery wedge pressure (PCW), and cardiac index (CI) were also measured before and during the 3 verapamil doses (V1-V3).

	C	V1	V2	V3
HR	76 ± 3	77 ± 2	81 ± 3*	81 ± 6
SBP	116 ± 4	108 ± 3*	107 ± 4*	99 ± 5*
PCW	14 ± 1	13 ± 1	15 ± 1	15 ± 1
CI	2.6 ± 0.1	2.7 ± 0.2	3.0 ± 0.3	2.8 ± 0.2†

†= $p<.05$ * = $p<.005$ (compared to C)

These results indicate that verapamil can significantly decrease left ventricular outflow tract obstruction in patients with HCM, and thus may provide an important new therapeutic agent in the treatment of this disorder.

Publications: Rosing, D.R., Kent, K.M., Borer, J.S., Seides, S.F., Maron, B.J. and Epstein, S.E.: Verapamil Therapy: A New Approach to the Pharmacologic Treatment of Hypertrophic tardiomyopathy: I. Hemodynamic Effects. Circulation, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01721-02 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Verapamil Effect on Exercise and Symptoms in 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
	Barry J. Maron	Senior Investigator	CB NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, 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 antagonist, was found to improve exercise capacity relative to placebo and subjective symptomatic status in a group of patients with hypertrophic cardiomyopathy, the majority of whom were medical failures with propranolol. The effect of verapamil on exercise capacity was similar to that of propranolol, but verapamil appeared to provide more subjective symptomatic benefit than propranolol.

PHS-6040
(Rev. 10-76)

Project Description: Presently, the primary approach to symptomatic therapy in patients with hypertrophic cardiomyopathy (HCM) is limited to beta blocking agents. We previously found that the Ca antagonist verapamil reduced left ventricular outflow obstruction of patients with HCM. To assess the clinical implications of this finding, treadmill exercise duration and symptomatic status were evaluated in 19 patients with hypertrophic cardiomyopathy during oral verapamil, propranolol and placebo administration. Drugs were given in a randomized double-blind fashion in two dosages (verapamil: 80 and 120 mg q6h, propranolol: 40 and 80 mg q6h). On placebo, exercise duration averaged 6.1±0.8 minutes with all patients stopped by angina, presyncope, or dyspnea. Exercise duration on verapamil increased by 26±8% (1.6±0.5 min, $p<.005$) and on propranolol by 21±8% (1.3±0.5 min, $p<.025$). Twelve patients on verapamil and 11 on propranolol improved by at least 15% compared to placebo. No patient on verapamil but three on propranolol had a decrease in exercise duration greater than 15% relative to placebo. Nine patients described their symptomatic status as "best" on verapamil, 7 did the same for placebo, and 3 for propranolol. Only four patients stated they felt "worst" on verapamil compared to 7 each for placebo and propranolol. Fifteen patients were discharged on chronic verapamil therapy. Four to 9 months after discharge exercise duration in eight patients on chronic verapamil improved 45± 15% ($p<.025$) relative to placebo and 21±5% ($p<.005$) relative to verapamil in hospital. Eleven patients reported symptomatic benefit with six improving their functional class by at least one grade. Our findings indicate that verapamil reduces left ventricular outflow obstruction and improves exercise capacity and symptomatic status in many patients with hypertrohic cardiomyopathy. Thus, verapamil offers a much needed new therapeutic approach to the pharmacologic treatment of this disorder.

Publications: Rosing, D.R., Kent, K.M., Maron, B.J., and Epstein, S.E.: A new approach to pharmacologic treatment of hypertrohic cardiomyopathy: II. Effects on Exercise Capacity and Symptomatic Status. Circulation, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01722-02 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Electrophysiology of Verapamil in Man: Dose-Response Relationship

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
	Stuart F. Seides	Senior Investigator	CB	NHLBI
	Robert O. Bonow	Senior Investigator	CB	NHLBI
	Lewis C. Lipson	Senior Investigator	CB	NHLBI
	Carolyn J. Ewels	Research Assistant	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:
.05	.03	.02

CHECK 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 is a potent inhibitor of A-V nodal transmission with little effect on other cardiac electrophysiologic functions.

PHS-6040
(Rev. 10-76)

Project Description: Verapamil is a new agent which is effective in the treatment of supraventricular arrhythmias and may prove to be effective in the hemodynamic treatment of hypertrophic cardiomyopathy. In order to assess the electrophysiologic effects of incremental doses of Verapamil, 14 patients with hypertrophic cardiomyopathy underwent intracardiac electrophysiologic study with programmed stimulation. All 14 patients received a bolus of 0.1 mg/kg followed by an infusion of 0.007 mg/kg/min; 10 patients received an additional bolus of 0.1 mg/kg and an infusion of 0.014 mg/kg/min; 6 patients received a third bolus of 0.1 mg/kg and an infusion of 0.021 mg/kg/min. There were no adverse effects and no patient had a decrease in systolic blood pressure exceeding 15 mmHg. Sinus cycle length and all parameters of A-V nodal function were progressively affected.

	Sinus CL	A-H interval+ (CL=600)	AVN-ERP+ (CL 600)	Paced CL at AVN block+
Control	813	103	211	364
0.007	775	129*	326*	428*
0.014	662	149*	407*	459*
0.021	600	173*	470*	500*

*p<05 + msec

Sinus node recovery time, QRS duration, Q-T interval, H-V interval, and atrial effective refractory period were not significantly affected at any dose level. Verapamil appears to be a potent and selective inhibitor of AVN conduction in man with a direct dose-response relationship.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01723-02 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Search for Left Main 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
	Robert O. Bonow	Senior Investigator	CB	NHLBI
	Lewis C. Lipson	Senior Investigator	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.1	.07	.03

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Coronary artery bypass surgery appears to prolong life of patients with left main coronary disease. Exercise testing can be used to identify such patients.

PHS-6040
(Rev. 10-76)

Project Description: Coronary revascularization has been shown to improve survival of symptomatic patients who have greater than 50% obstruction of the left main coronary artery. Thus, it is imperative to identify the subset of individuals who have left main coronary artery obstruction. The prevalence of left main coronary artery disease is approximately 10 to 15 percent of all patients with coronary artery disease. In 300 consecutive patients with coronary artery disease evaluated at the N.I.H., 140 were functional class I or II (asymptomatic or mild symptoms) and 160 patients were functional class III or IV (severe symptoms). Left main coronary artery disease ($\geq$50% reduction in diameter) was present in 40 patients (overall prevalence 13%) with 14 patients being asymptomatic or mildly symptomatic (10%) and 26 patients being more severely symptomatic (16%). Thus, left main coronary artery disease may be present in patients who either have no symptoms or mild symptoms with a prevalence of approximately 10%. There were no distinguishing clinical characteristics to allow the identification of patients with left main coronary artery disease. However, graded exercise testing to angina or 85% of the maximal predicted heart rate demonstrated that 93% of our patients with left main coronary artery dsiease had 1mm segement ST depression or greater as compared to 48% of patients without left main coronary artery disease ($p<.01$). Of the asymptomatic or mildly symptomatic patients 13/14 had $\geq$1mm ST segment depression as compared to 50% of asymptomatic or mildly symptomatic patients without left main coronary artery disease. Thus graded exercise testing to either angina or 85% predicted maximum heart rate identifies most all patients with left main coronary artery disease and eliminates approximately 50% of the patients without left main coronary artery disease. These results suggest that exercise ECG testing can be used to select patients for coronary angiography. Selecting only patients with a positive ECG test would result in a probability of greater than 90% of identifying all patients with left main coroanry artery disease and would eliminate the need for coronary angiography in approximately half of the patients with symptomatic coronary artery disease. It is important to note that this was a submaximal stress test. If patients without left main coronary artery disease were stressed to their maximum tolerance, many more patients would have positive ($\geq$1mm ST segment depression) tests, resulting in considerable decrease in the predictive accuracy for a positive test being indicative of left main disease.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01724-02 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Ventricular Septal Disorganization 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:	William C. Roberts	Chief, 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:	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)

A quantitative method was devised to determine the extent to which disorganized cardiac muscle cells are present in the ventricular septum of patients with hypertrophic cardiomyopathy. Extensive cellular disorganization proved to be a highly specific and sensitive marker of hypertrophic cardiomyopathy.

PHS-6040
(Rev. 10-76)

Project Description: The presence of numerous abnormally arranged cardiac muscle cells in the ventricular septum has been considered a characteristic anatomic feature of patients with hypertrophic cardiomyopathy. To determine the specificity of this histologic marker for patients with hypertrophic cardiomyopathy, we used a quantitative method to determine the area of myocardium occupied by disorganized cells. In hypertrophic cardiomyopathy, septal disorganization was present in 94% of the 54 patients studied at necropsy. Furthermore, disorganization was extensive in most of these patients, involving 5% or more of the transverse plane tissue section in 89% of the patients and 25% or more of the section in 56% of the patients. Septal disorganization was best identified in tissue sections cut perpendicular to the long axis of the left ventricle. Septal disorganization was present in only 26% of the 144 control patients with other heart diseases or normal hearts. Most important, when present in these patients, disorganization was usually limited in extent. In only 7% of the controls studied did abnormally arranged cells occupy 5% or more of the tissue section. The average area of septum disorganized was $31 \pm 3\%$ (mean $\pm$ SEM) in patients with hypertrophic cardiomyopathy and only $1.5 \pm 0.6\%$ in the controls ($p < 0.001$). Hence, while the presence of ventricular septal disorganization is not pathognomonic of hypertrophic cardiomyopathy, widespread distribution of this abnormality is a very sensitive and specific histologic marker for this disease.

Publications: Maron, B.J. and Roberts, W.C.: Quantitative analysis of cardiac muscle cell disorganization in the ventricular septum of patients with Hypertrophic cardiomyopathy. Circulation 59: 689-706, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01725-02 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Disproportionate Septal Thickening in Infants with Congenital Heart Diseases

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:	Jesse E. Edwards	Chairman, Dept.of Pathology-United Hosp.-Miller Div.	
	James H. Moller	Chairman, Dept.of Pediatrics-Univ.of Minn.Sch.of Med.	
	Stephen E. Epstein	Chief, Cardiology Branch	CB NHLBI

COOPERATING UNITS (if any)
Dept. of Pathology - United Hospitals - Miller Division, St. Paul, Minn.
Dept. of Pediatrics - University of Minnesota School of Medicine.

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)

A disproportionate thickened ventricular septum is a relatively common finding (prevalence about 25%) in infants with a variety of congenital heart diseases. Such abnormal septal-free wall ratios are probably a function of small differences between septal and free thicknesses in hearts with relatively thin ventricular walls. An abnormal septal-free wall ratio cannot be used as the sole criterion for identifying hypertrophic cardiomyopathy in infants with other heart diseases.

PHS-6040
(Rev. 10-76)

Project Description: Necropsy studies were performed in 125 infants (two years of age or less) with a variety of congenital heart malformations. Disproportionate ventricular septal thickening (septal to left ventricular free wall thickness ratio $\geq$1.3) was present in 31 (25%) of the 125 patients. In the vast majority of patients, an abnormal septal-free wall ratio did not appear to be a manifestation of genetically transmitted hypertrophic cardiomyopathy since: 1) marked disorganization of septal myocardium (involving $\geq$5% of the tissue section) was present in only 5 of the 31 patients with disproportionate septal thickening; and 2) echocardiographic studies in first degree relatives of four other patients with disproportionate septal thickening and normal septal architecture did not show asymmetric septal hypertrophy. The relatively high prevalence of septal-free wall ratios of 1.3 or greater in a population of infants with congenital heart disease appears to be due, in part, to the fact that minor differences in ventricular wall thicknesses (in patients with relatively thin absolute wall thicknesses) may produce particularly large deviations from unity in septal-free wall ratio. Hence, 1) non-genetically transmitted disproportionate septal thickening is relatively common in infants with congenital heart diseases studied at necropsy; and 2) septal free wall ratio of $\geq$1.3 cannot be used as the sole criterion for identifying associated hypertrophic cardiomyopathy in infants with other congenital heart diseases, particularly if marked absolute septal thickening is absent.

Publications: Maron, B.J., Edwards, J.W., Moller, J.H., and Epstein, S.E.: Prevalence and Characteristics of Disproportionate Ventricular Septal Thickening in Infants with Congenital Heart Diseases. Circulation 59: 126-133, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01727-02 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Septal Disorganization as Specific Marker for 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:	Noriko Sato	Guest Worker	CB NHLBI
	William C. Roberts	Chief, Pathology Branch	PB NHLBI
	Jesse E. Edwards	Chairman, Dept. of Pathology-United Hosp.-Miller Div.	
	Roma S. Chandra	Staff Pathologist-Children'n Hosp.Med.Center	

COOPERATING UNITS (if any)
Pathology Branch, NHLBI
United Hospitals- Miller Division, St. Paul, Minn.
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:	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)

Marked disorganization of cardiac muscle cells in the ventricular septum is a highly specific (as well as sensitive) marker for hypertrophic cardiomyopathy. Although septal disorganization may occur in patients with aortic or pulmonic atresia (as well as other congenital heart malformations) the areas of septum involved are usually extremely small.

PHS-6040
(Rev. 10-76)

Project Description: The presence of numerous abnormally arranged cardiac muscle cells in the ventricular septum has been considered to be a characteristic anatomic feature of patients with hypertrophic cardiomyopathy. However, it has been suggested that the ventricular septum of infants with certain congenital cardiac diseases (such as aortic or pulmonic valve atresia) contains disorganized cardiac muscle cells similar to that present in patients with hypertrophic cardiomyopathy. To test the validity of this concept and the true specificity of septal disorganization for hypertrophic cardiomyopathy, sections of ventricular septum were obtained at necropsy from 276 patients and the extent of ventricular septal disorganization was determined quantitatively. Disorganization was most marked in infants, children and adults with hypertrophic cardiomyopathy - i.e., present in 95% of 60 patients; the mean area of septum disorganized was 31 $\pm$ 3%. Although disorganized cells were present in 64% of 33 infants with aortic or pulmonic valve atresia, these cells occupied extremely small areas of ventricular septum (mean area of septum disorganized 2.8 $\pm$ 0.7%; $p<0.001$). Furthermore, the minimal septal disorganization present in aortic or pulmonic valve atresia was similar to that found in 91 infants with other congenital heart malformations (mean area disorganized 1.4 $\pm$ 0.6%), and in 92 normal fetuses or infants (mean area disorganized 0.3 $\pm$ 0.1%). Hence, extensive ventricular septal disorganization is a highly sensitive and specific finding for hypertrophic cardiomyopathy, although small areas of disorganization may occur in infants with a variety of other heart diseases, including aortic or pulmonic valve atresia.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01730-02 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Sensitivity of Radionuclide Cineangiography and Thallium Scanning in C.A.D.

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

PI:	J.S. Borer	Senior Investigator	CB	NHLBI
Other:	S.L. Bacharach	Physicist	NM	CC
	M.V. Green	Chief, Applied Physics Sec.	NM	CC
	J.J. Bailey	Chief, Med. Appls. Sec., DCRT	LAS	DCRT
	K.M. Kent	Head, Cardiovascular Diagnosis	CB	NHLBI
	D.R. Rosing	Senior Investigator	CB	NHLBI
	S.F. Seides	Senior Investigator	CB	NHLBI
	S.E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)
Nuclear Medicine Dept., CC
Division of Computer Research and Technology, NIH.

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.8	.6	.2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Radionuclide cineangiography and Thallium perfusion scanning both are known to be sensitive indicators of the presence of coronary artery disease. We employed both techniques in patients with known or suspected coronary artery disease to permit comparison of the accuracy of the two techniques in the diagnosis of coronary artery disease. We found that both radionuclide cineangiography and perfusion scanning with Tl^{201} are sensitive in diagnosis of coronary artery disease, but radionuclide cineangiography also offers potentially valuable quantitative assessment of functional severity of coronary artery disease.

179

Project Description: Evaluation of global and regional left ventricular function with Tc^{99m} radionuclide cineangiography (RC) and evaluation of regional perfusion with Tl^{201} scanning (PS) both have proven accurate in detecting patients with coronary artery disease (CAD). To compare the sensitivity of these methods, 74 patients with suspected CAD were studied 1) at rest and during maximal exercise with RC, and 2) immediately after identical exercise, and 2-3 hrs post-exercise with PS, using 3 view and 4 different background subtraction schemes. Fifty-eight of the 74 patients had angiographically demonstrable $\geq$50% occlusion of $\geq$1 coronary artery; 16 had normal coronary arteries. Fifty-four of the 58 CAD patients (93%) had diagnostic RC regional and global abnormalities; in >90% of the patients ejection fraction (EF) fell with exercise (EF rest = 49 ± 3%, exercise = 42 ± 3%, $p<.001$); 47 of 58 (81%) had PS abnormalities. The difference in sensitivity was significant ($p<.05$) and no patient had an abnormal PS without an abnormal RC. Of the 16 patients without CAD, 0 had RC abnormalities and 2 had a PS abnormality. Thus, both RC and PS are sensitive in diagnosis of CAD, but RC also offers potentially valuable quantitative assessment of functional severity of CAD.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01731-02 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Left Ventricular Function in Aortic Stenosis: Effects of Operation

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

PI:	Jeffrey S. Borer	Senior Investigator	CB	NHLBI
Other:	Stephen L. Bacharach	Physicist	NM	CC
	Michael V. Green	Chief, Applied Physics Sec.	NM	CC
	Kenneth M. Kent	Head, Cardiovascular Diagnosis	CB	NHLBI
	Douglas R. Rosing	Senior Investigator	CB	NHLBI
	Stuart F. Seides	Senior Investigator	CB	NHLBI
	Charles L. McIntosh	Senior Surgeon	SB	NHLBI
	David M. Conkle	Senior Surgeon	SB	NHLBI
	Andrew G. Morrow	Chief, Surgery Branch	SB	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:
.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)

In patients with aortic stenosis routine preoperative hemodynamic assessment does not accurately predict long-term post-operative prognosis. Such assessments are performed with the patient at rest. Therefore, we used radionuclide cine-angiography during exercise to assess left ventricular functional reserve capacity to determine accurately the functional status of the left ventricle, the relationship of ventricular function during exercise to prognosis and the effects of aortic valve replacement on left ventricular function. We found that, while left ventricular function commonly is depressed during exercise before operation, function almost invariably returns to normal during exercise after operation, irrespective of the severity of the previously present valvular stenosis.

181

Project Description: In patients with severe aortic stenosis (AS), preoperative (op) clinical and hemodynamic assessment has not accurately predicted long-term prognosis after operation. However, such studies have been performed with the patient at rest. Study during the stress of exercise, by unmasking abnormalities in left ventricular function (LVF), might provide a more useful prognostic index, and also might permit accurate evaluation of the effects of operation on LVF. Therefore, we have utilized non-invasive radionuclide cineangiography to assess LVF during the stress of intense exercise. Twenty-eight patients with severe AS were studied. Prior to operation ejection fraction (EF) at rest was $68\pm3\%$, significantly greater than normal ($57\pm1\%$, $p<.001$). During exercise preop, however, EF fell to $57\pm4\%$ (nl $71\pm2\%$, $p<.001$). Moreover, during exercise all 30 normal subjects studied increased EF, and none had EF <55%; EF fell in 25 of the 28 patients with AS, and was <55% in 14. Exercise EF could not be predicted from rest EF, aortic valve area, gradient, or LVEDP. Thus far 20 patients have returned 6 mos postop. Postop EF at rest ($68\pm5\%$) was unchanged, but during exercise it rose significantly from $56\pm7\%$ to $72\pm3\%$ ($p<.05$). We conclude that while high normal EF is present at rest in most patients with severe AS, the stress of exercise causes a marked fall in EF in some patients. However, while operation often does not change the already supernormal EF at rest, it significantly improves EF response during exercise. This result is very different from that found in patients with aortic regurgitation whose exercise EF usually does not return to normal after operation. These findings suggest that myocardial dysfunction after operation is very much less in patients with AS than with AR, and further suggests that long term prognosis might be expected to be better in patients with AS than with AR.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01733-02 CB

PERIOD COVERED
October 1, 1978, to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Effect of Operation on Left Ventricular Function in Patients with IHSS

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

PI:	J.S. Borer	Senior Investigator	CB	NHLBI
Other:	S.L. Bacharach	Physicist	NM	CC
	M.V. Green	Chief, Applied Physics Sec.	NM	CC
	D.R. Rosing	Senior Investigator	CB	NHLBI
	K.M. Kent	Head, Cardiovascular Diagnosis	CB	NHLBI
	A.G. Morrow	Chief, Surgery Branch	SB	NHLBI
	S.E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)
Surgery Branch, NHLBI

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.6	.5	.1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Patients with hypertrophic subaortic stenosis usually have supernormal left ventricular function at rest and normal function during exercise despite the presence of symptoms requiring surgical intervention. We used radionuclide cineangiography at rest and during exercise to assess the effects of septal myotomy and myectomy, which involves the removal of moderate amounts of myocardium on left ventricular function. We found the operation usually does not importantly impair left ventricular function, at rest or during exercise stress.

183

Project Description: Patients with idiopathic hypertrophic subaortic stenosis (IHSS) have supernormal left ventricular systolic function at rest. Septal myotomy/myectomy reduces outflow obstruction and symptoms. However, it has been proposed that operation causes myocardial damage, with consequent reduction in left ventricular function and potentially deleterious long-term consequences, despite symptom relief. To evaluate the effect of myotomy and myectomy on left ventricular function we employed non-invasive radionuclide cineangiography at rest and during maximal exercise in 21 consecutive patients with IHSS, before and 6 months after operation. In 30 normal subjects, ejection fraction (EF) was $57\pm2\%$ at rest and $71\pm2\%$ during exercise. Before operation in patients with IHSS, EF was supernormal at rest ($76\pm2\%$, $p<.001$), and normal during exercise ($71\pm3\%$, NS). All patients improved from NYHA functional class III-IV preop to class I-II postop; maximal provocable gradient invariably diminished (av. 100 ± 8 mmHg preop, 33 ± 9 mmHG postop, $p<.001$). Postop, EF at rest was $67\pm2\%$, still supernormal but less than preop ($p<.001$). During exercise EF was $66\pm4\%$, only mildly diminished ($p<.01$) from preop. We conclude that muscle resection during myotomy and myectomy usually does not importantly impair left ventricular function at rest or during exercise stress.

Publications: Borer, J.S., Bacharach, S.L., Green, M.V., Kent, K.M., Rosing, D.R., Seides, S.F., Morrow, A.G., and Epstein, S.E.: Effect of septal myotomy and myectomy on left ventricular systolic function at rest and during exercise in patients with I.H.S.S. Circulation , in press

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01734-02 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Echo Assessment of Cardiac Anatomy and Function in Acromegalic Patients

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

PI:	Daniel D. Savage	Senior Staff Fellow	CB	NHLBI
Other:	Walter L. Henry	Senior Investigator	CB	NHLBI
	R. Eastman	Clinical Associate	DB	NIAMDD
	Jeffrey S. Borer	Senior Investigator	CB	NHLBI
	Phillip Gorden	Senior Investigator	DB	NIAMDD

COOPERATING UNITS (if any)
NIAMDD (Diabetes Branch), NIH.

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.04	.02	.02

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Cardiovascular complications are among the most common causes of morbidity and mortality in patients with acromegaly. Echocardiography was used to characterize the prevalence and types of anatomic and functional abnormalities of the heart in such patients. The most common abnormality was increased left ventricular mass with concentric left ventricular wall thickening, which was found in 64% (16/25) of patients.

185

Project Description: Echocardiography was used to evaluate cardiac anatomy and function in 25 patients with acromegaly. Eighty percent of the patients (20/25) had abnormal echocardiograms including 13 patients without other signs or symptoms of cardiac involvement. The most common abnormality was increased left ventricular mass with concentric left ventricular wall thickening, which was found in 64% (16/25) of patients. Increased left ventricular transverse dimension at end-diastole was found in 36% (9/25) of patients. Other echocardiographic abnormalities found included left atrial or aortic root enlargement (two patients), disproportionate septal thickening (one patient) and depressed left ventricular ejection fraction (three patients). Since six of the patients with increased left ventricular mass had no history of hypertension or hyperthyroidism, the increased mass cannot be ascribed solely to these conditions commonly associated with acromegaly. Furthermore, the degree of cardiac hypertrophy showed no significant correlation with pre- or post-treatment growth hormone levels or known duration of acromegaly prior to treatment. Thus, although the pathophysiology and significance of cardiac abnormalities in acromegalic patients is not yet fully understood, echocardiography does provide a sensitive tool for detecting such abnormalities before they are otherwise apparent.

Publications: Savage, D.D., Henry, W.L., Eastman, R., Borer, J.S., and Gorden, P.: Echocardiographic assessment of cardiac anatomy and function in acromegalic patients. Amer J Med, November, 1979, In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01737-02 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Functional Adaptations of the Left Ventricle to Chronic Aortic Regurgitation

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

PI:	Walter L. Henry	Senior Investigator	CB	NHLBI
Other:	Jeffrey S. Borer	Senior Investigator	CB	NHLBI
	Robert O. Bonow	Senior Investigator	CB	NHLBI
	Douglas R. Rosing	Senior Investigator	CB	NHLBI
	Stephen L. Bacharach	Physicist	NM	CC
	Michael V. Green	Chief, Applied Physics Sec.	NM	CC
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)
Nuclear Medicine, Clinical Center, NIH.

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.1	.06	.04

CHECK 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 relationship between left ventricular (LV) volume overload and LV systolic function in chronic aortic regurgitation (AR), 80 patients were studied by both echocardiography at rest and radionuclide cineangiography at rest and during exercise. LV fractional shortening remained within the normal range until LV diastolic dimension by echo exceeded 65 mm and LV systolic dimension exceeded 42 mm (both representing a 40% increase above expected normal values). Above these dimensions, systolic dimension increased disproportionately greater than diastolic dimension, and fractional shortening fell progressively. Similarly radionuclide cineangiographic LV ejection fraction (EF) at rest and during exercise was normal in patients with LV dimensions increased up to 40% above normal. Above this value, EF was often normal at rest, but was subnormal in 96% of patients. Thus, the LV can increase its internal dimensions approximately 40% and maintain normal systolic function. Above this point further dilatation is accompanied by a progressive LV systolic dysfunction.

157

Project Description: Although many patients are able to adapt to the volume overload produced by chronic aortic regurgitation (AR), others develop depression of left ventricular (LV) systolic function (SF). To assess the relationship between LV volume overload and LVSF, we have studied 80 patients with chronic AR with echocardiography at rest and with radionuclide cineangiography at rest and during exercise. While all patients had some degree of LV dilatation by echocardiography, fractional shortening remained within normal limits until both 1) LV end-diastolic dimension exceeded 65 mm and 2) LV end-systolic dimension exceeded 42 mm. These dimensions were 40% greater than the upper limit expected for these patients body surface areas, and correspond to a 250% increase in estimated LV volumes. In patients with LV dimensions >40% above normal, end-systolic dimension increased to a disproportionately greater extent than end-diastolic dimension; fractional shortening fell progressively, in proportion to the increase in each of the ventricular dimensions. Similarly, radionuclide cineangiographic LV ejection fractions (EF) at rest and during exercise were normal in patients with LV dimensions increased up to 40% above normal. Above this value, though EF at rest often was normal, EF during exercise was subnormal in 25 of 26 (96%) patients. Thus, both echo and radionuclide data demonstrate that the LV can increase its internal dimensions approximately 40% and maintain normal systolic function. At this point, the functional reserve of the LV is exceeded and further dilatation is accompanied by a progressive fall in LV systolic function.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01742-01 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Left Ventricular Functional Reserve in Adult Patients with Atrial Septal Defect

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

PI:	Robert O. Bonow	Senior Investigator	CB NHLBI
Other:	Jeffrey S. Borer	Senior Investigator	CB NHLBI
	Douglas R. Rosing	Senior Investigator	CB NHLBI
	Kenneth M. Kent	Head, Cardiovascular Diagnosis	CB NHLBI
	Michael V. Green	Chief, Applied Physics Sec.	NM CC
	Stephen L. Bacharach	Physicist	NM CC
	Stephen E. Epstein	Chief, Cardiology Branch	CB NHLBI

COOPERATING UNITS (if any)

Nuclear Medicine, Clinical Center, NIH.

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.1	.08	.02

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Radionuclide cineangiography at rest and during exercise was utilized to assess left ventricular function in eight patients with atrial septal defect (ASD). All patients had normal left ventricular ejection fraction (LVEF) at rest, but during exercise, four patients had subnormal increments in LVEF and two other patients manifest a decrease in LVEF below 55%, the lower limit of normal. These two patients had exertional dyspnea and orthopnea and also had significantly higher pulmonary to systemic flow ratios than the other 6. After operation, all patients are asymptomatic and all have normal LVEF both at rest and during exercise. Thus, left ventricular dysfunction in patients with ASD is due in part to reversible mechanical factors rather than to intrinsic, irreversible myocardial dysfunction.

PHS-6040
(Rev. 10-76)

Project Description: Controversy exists regarding the status of left ventricular (LV) function in patients with atrial septal defect (ASD). To assess LV function we used radionuclide cineangiography at rest and during exercise in 8 patients with ASD (ages 16-59, mean 36). No patient had other cardiac abnormalities. All patients had normal LV ejection fraction (EF) at rest (mean 62% ± 3% SE vs normal 57% ± 1%; NS); during exercise 2 or 8 increased LVEF normally, 4 had no change in LVEF with exercise, and in 2 LVEF decreased below 55%, the lower limit of normal. These 2 patients had exertional dyspnea and orthopnea; the other 6 were asymptomatic. These 2 patients also had significantly higher pulmonary to systemic flow ratios (Qp/Qs) than the other 6 patients (mean 4.8 vs 1.8, $p < .001$) but did not differ with respect to age, other hemodynamic data, or echo data. For all patients, significant correlations existed between the change in LVEF from rest to exercise and the magnitude of Qp/Qs ($r = .78$, $p < .05$). All patients had abnormalities of ventricular septal motion on echocardiography with subnormal LV diastolic dimensions secondary to the right ventricular volume overload. After operation, all 8 patients are asymptomatic, and all have normal rest and exercise LVEF (mean EF 59 ± 3% rest, 66 ± 4% exercise, $p < .01$), including the 2 patients with abnormal preop exercise LVEF. These data suggest that diminished LV functional reserve in adult patients with ASD is related to the magnitude of Qp/Qs and may be reversed by eliminating the shunt. Moreover, LV diastolic dimension by echo increased into the normal range in all patients postop. We conclude that LV dysfunction in many cases is due in part to reversible mechanical factors related to right ventricular volume overload rather than to intrinsic, irreversible myocardial dysfunction.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01743-01 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Aortic Valve Replacement for Combined Aortic Valvular and Coronary Artery Disease

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

PI:	Robert O. Bonow	Senior Investigator	CB	NHLBI
Other:	Kenneth M. Kent	Head, Cardiovascular Diagnosis	CB	NHLBI
	Douglas R. Rosing	Senior Investigator	CB	NHLBI
	Lewis C. Lipson	Senior Investigator	CB	NHLBI
	Charles L. McIntosh	Senior Surgeon	SB	NHLBI
	Andrew G. Morrow	Chief, Surgery Branch	SB	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 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.06	.04	.02

CHECK 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 test the hypothesis that routine coronary artery bypass surgery (CABG) is not required in patients undergoing aortic valve replacement (AVR) who have coexistent coronary artery disease (CAD), 51 patients with CAD underwent AVR without CABG since 1972. Another 145 patients without CAD underwent AVR during the same period. In patients with CAD, operative mortality (4%) and late mortality (22% at 3 years) were not significantly different from those without CAD. Moreover, only 14% of patients with CAD had myocardial infarctions during postoperative follow up, (avg., 3.0 years) and 8% subsequently required CABG because of angina. These data suggest that preoperative detection of CAD does not warrant routine CABG in all patients at the time of AVR.

PHS-6040
(Rev. 10-76)

To test the hypothesis that routine coronary artery bypass surgery (CABG) is not required in patients undergoing aortic valve replacement (AVR) who have coexistent coronary artery disease (CAD), 51 symptomatic patients with CAD underwent AVR without CABG since 1972; 26 had aortic stenosis (AS), 10 had aortic regurgitation (AR), and 15 had combined AS/AR. Another 145 patients without CAD underwent AVR for AS (52 patients), AR (54 patients) or AS/AR (39 patients) during the same period. Operative mortality was 4% in patients with CAD and 6% in patients without CAD. Moreover, late survival was not significantly different between the two groups when analyzed for the entire population (77% survival at 3 years in CAD patients, 80% in non-CAD patients) or for the subpopulation of AS, AR, or AS/AR. Seven of the 51 CAD patients (14%) had myocardial infarctions during postop follow-up (17 to 68 months), and 4 (8%) required CABG because of angina (12-43 mos). Thus, operative mortality and incidence of CAD complications postop is low in patients with associated CAD when AVR is performed without CABG. Moreover, long-term postop survival is comparable to patients without CAD. These data suggest that preop detection of CAD does not warrant CABG in all patients at the time of AVR.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01744-01 CB

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Effect of Nitroglycerin in Patients with Aortic Regurgitation and LV Dysfunction

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

PI:	Robert O. Bonow	Senior Investigator	CB	NHLBI
Other:	Jeffrey S. Borer	Senior Investigator	CB	NHLBI
	Douglas R. Rosing	Senior Investigator	CB	NHLBI
	Stephen L. Bacharach	Physicist	NM	CC
	Michael V. Green	Chief, Applied Physics Sec.	NM	CC
	Kenneth M. Kent	Head, Cardiovascular Diagnosis	CB	NHLBI

COOPERATING UNITS (if any)

Nuclear Medicine Department, CC, NIH.

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.05	.04	.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)

Nitroglycerin (TNG) was administered to 15 patients with aortic regurgitation and left ventricular (LV) dysfunction determined by radionuclide cineangiography during supine exercise. TNG resulted in a 25% decrease in LV end-diastolic volume (LVEDV) during exercise ($p<.01$) with no change in exercise heart rate; TNG also resulted in a significant increase in exercise LV ejection fraction ($40\pm10\%$ pre-TNG to $47\pm10\%$ post-TNG). Thus, despite reduction in LVEDV (causing reduction in wall stress), stroke volume diminished only 5% at maximal exercise. The TNG-induced reduction in LV wall stress with maintenance of external work may be of value in the long-term management of patients with aortic regurgitation.

PHS-6040
(Rev. 10-76)

Project Description: To determine the effect of nitroglycerin (TNG) in patients with aortic regurgitation and depressed left ventricular function, we utilized radionuclide cineangiography during exercise before and after TNG in 15 patients. Prior to TNG, all had subnormal ejection fraction either at rest (<45%, 5 patients) or with exercise (<55%, 15 patients). After TNG in doses sufficient to diminish systolic arterial pressure >10 mmHg and/or increase heart rate >10 beats/min, left ventricular end-diastolic volume (LVEDV) fell 18% at rest and 25% during exercise (both $p<.01$ compared with pre-TNG values). TNG caused no change in resting ejection fraction ($50\pm7\%$ pre-TNG vs $53\pm9\%$ post-TNG, NS); however, ejection fraction increased significantly during exercise ($40\pm10\%$ pre-TNG to $47\pm10\%$ post-TNG, $p<.001$). Exercise heart rate was unchanged. Despite reduction in LVEDV (causing reduction in wall stress), stroke volume diminished only 5% at maximum exercise and an increase in left ventricular ejection fraction during exercise in patients with aortic regurgitation and impaired left ventricular function. The TNG-induced reduction in left ventricular wall stress with maintenance of external work may be of value in the long-term clinical management of patients with aortic regurgitation.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01745-01 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Detection of Cardiac Dysfunction in Patients with Transfusion-Dependent Anemia

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

PI:	Martin B. Leon	Clinical Associate	CB	NHLBI
Other:	Jeffrey S. Borer	Senior Investigator	CB	NHLBI
	Stephen L. Bacharach	Physicist	NM	CC
	Michael V. Green	Chief, Applied Physics Section	NM	CC
	Arthur W. Nienhuis	Chief, Hematology Branch	HB	NHLBI

COOPERATING UNITS (if any)
Nuclear Medicine Department, CC, NIH.
Hematology Branch, NHLBI

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

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)

The identification of early cardiac dysfunction in patients with systemic iron overload is critical in assessing the efficacy of iron removal therapy. In 24 patients with transfusion-dependent, congenital anemia radionuclide cineangiography at rest and during exercise was shown to be a highly sensitive technique for detecting pre-clinical myocardial dysfunction and may be useful in evaluating iron removal theraphy.

PHS-6040
(Rev. 10-76)

Project Description: Patients with transfusion-dependent congenital anemia and secondary myocardial iron deposition usually die from progressive refractory congestive heart failure. Iron removal therapies may potentially reverse this course, but accurate assessment of subtle changes in left ventricular performance are necessary to document the therapeutic efficacy of such modalities. Therefore, we used radionuclide cineangiography to determine left ventricular ejection fraction during exercise and regional functional abnormalities in 24 patients, age 8-30 years, with transfusion-dependent, congenital anemia. Ejection fraction at rest was normal in 21 patients; however, during exercise, ejection fraction was normal in only 11 patients. All 8 patients receiving <100 transfusions had normal exercise ejection fraction, whereas only 3 of 16 patients with ≥100 transfusions had normal exercise ejection fraction. Echocardiographic fractional shortening was normal in 16 of 19 patients studied and all patients with subnormal fractional shortening had subnormal exercise ejection fraction at rest, and normal fractional shortening by echo, exercise ejection fraction was subnormal. Regional functional abnormalities were detected in 3 patients, all with ≥100 transfusions and subnormal exercise ejection fraction. Thus, radionuclide cineangiography is a highly sensitive technique for detecting preclinical myocardial dysfunction in patients with chronic iron overload and may be useful in assessing iron removal therapies.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01746-01 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Sulfinpyrazone Increases Collateral Blood Flow Following Acute Coronary Occlusion

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

PI:	Nancy Davenport	Post-Doctoral Fellow	CB	NHLBI
Other:	Robert E. Goldstein	Senior Investigator	CB	NHLBI
	Norine L. Capurro	Senior Staff Fellow	CB	NHLBI
	N. Raphael Shulman	Chief, Clin. Hematology Br.	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)
Clinical Hematology Branch, NIAMDD, NIH.

LAB/BRANCH
Cardiology Branch

SECTION
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.8	.7	.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)

Sulfinpyrazone, a drug known to inhibit platelet aggregation and prostaglandin synthesis, was found to increase collateral flow to ischemic canine myocardium. Epicardial collateral flow increased 35% five minutes after coronary occlusion and 68% four hours after occlusion as compared to flow during a temporary occlusion before the drug was given. Very small increases in endocardial collateral flow also occurred in the sulfinpyrazone treated animals. The dose of sulfinpyrazone used, 30 mg/kg, was shown to completely inhibit platelet aggregation. The reported beneficial action of sulfinpyrazone in coronary artery dsiease may be related to drug effects on collateral flow.

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Project Description: Recent studies suggest that daily administration of sulfinpyrazone (SPZ) decreases patient mortality during the first year following acute myocardial infarction (AMI). However, the effect of SPZ, a known platelet-inhibiting drug, immediately following acute coronary occlusion is unknown. Previous studies of two other platelet inhibitors, aspirin and indomethacin, demonstrated that myocardial collateral blood flow (CBF) is increased by aspirin but decreased by indomethacin. To determine whether changes in CBF are induced by SPZ, 21 open-chest dogs were studied. Radioactive microspheres (15±5μ) were injected 5 min after occlusion of left anterior descending (LAD) coronary artery. The occlusion was then released and SPZ 30 mg/kg administered to 11 dogs; 10 control dogs received saline. One hr after release the LAD was permanently occluded and microspheres injected 5 min and 4 hrs thereafter. No significant changes in CBF to normal or ischemic zones occurred in control dogs. In the ischemic zone epicardium of the SPZ treated group, CBF, which was 0.13 ml/min/g 5 min after first occlusion, rose to 0.20 ($p \leq .01$) 5 min after 2nd occlusion and to 0.31 ($p \leq .02$) 4 hrs after 2nd occlusion. Small changes occurred in the ischemic zone endocardium: CBF, 0.035 5 min after 1st occlusion, rose to 0.084 ($p \leq .01$) 5 min after 2nd occlusion and remained elevated (0.076) 4 hrs after 2nd occlusion. The platelets of each SPZ-treated dog showed a completely inhibited response to ADP-induced aggregation. Thus SPZ, like aspirin, can increase CBF to ischemic epicardium. This favorable effect on CBF may play a role in the salutary action of SPZ in patients following AMI.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01747-01 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Instability of Microsphere Content in Acutely Ischemic Myocardium

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

PI:	Nancy Davenport	Post-Doctoral Fellow	CB	NHLBI
Other:	Robert E. Goldstein	Senior Investigator	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:
.2	.15	.05

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Decrease of microsphere in ischemic tissue over time poses a serious problem to this method of blood flow measurement. Myocardial water content was studied to determine the extent to which edema contributed to the decreased microsphere content. After four hours of occlusion, a 21% decrease in preocclusion microsphere content was noted in the ischemic endocardium. While the same tissue showed a 3% increase in water content, the amount of edema and extent of microsphere decrease were not correlated. Alterations in microsphere content cannot be corrected by measurement of myocardial water content.

PHS-6040
(Rev. 10-76)

Project Description:

Ischemic zone (IZ) microsphere content per unit wet weight is decreased 24 hr after coronary occlusion suggesting limitations in the use of microspheres. To evaluate rapidity of this "loss" and the contribution of altered water content, we injected 15μ radioactive microspheres before and after left anterior descending coronary artery occlusion in 11 dogs. Ischemia was confirmed by low postocclusion flows. Animals were sacrificed 4 hr after ligation and myocardial samples weighed before and after lyophilization. In 4 nonischemic controls microsphere distribution was uniform in the left ventricle. Preocclusion endocardial (Endo) flow values were .26 ml/min/g (21%) lower in IZ than in normally perfused zone (NZ) ($p<.01$); water content of IZ Endo was higher than NZ Endo (80.3 vs 77.0%, $p<.001$). Although water content of IZ epicardium (Epi) was also higher than NZ Epi (79.2% vs 75.2%, $p<.001$), preocclusion Epi flows were .18 ml/min/g (15%) higher in IZ than in NZ ($p<.005$). Rise in IZ Endo water content was not correlated with microsphere "loss". Rise in tissue water was correlated ($p<.01$) with fall in postocclusion flow in both Epi ($r=.72$) and Endo ($r=.76$). Thus myocardial microsphere content and tissue water are altered significantly after 4 hrs of ischemia. However, correction of microsphere content based upon water content does not appear feasible.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01748-01 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Ibuprofen and Sulfinpyrazone Inhibit Prostaglandin-Mediated Coronary Vasodilation

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

PI:	Robert E. Goldstein	Senior Investigator	CB	NHLBI
Other:	Nancy Davenport	Post-doctoral Fellow	CB	NHLBI
	Lewis C. Lipson	Senior Investigator	CB	NHLBI
	Robert O. Bonow	Senior Investigator	CB	NHLBI
	Norine Capurro	Senior Staff Fellow	CB	NHLBI
	N. Raphael Shulman	Chief,Clin.Hematology Branch	CHM	NIAMDD
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)
Clinical Hematology Branch, NIAMDD, NIH.

LAB/BRANCH
Cardiology Branch

SECTION
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.4	.3	.1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The ability of various doses of sulfinpyrazone and ibuprofen to inhibit prostaglandin-mediated coronary vasodilation and to inhibit platelet aggregation was evaluated in dogs. Doses of these drugs that uniformly inhibited platelets also markedly reduced vasodilator responses to the prostaglandins precursor arachidonic acid. Threshold doses for inhibition of coronary vasodilation and platelet aggregation were also similar. We conclude that doses of sulfinpyrazone or ibuprofen sufficient to interfere with platelet aggregation are also likely to inhibit prostaglandin-mediated coronary vasodilation, possibly through disruption of prostaglandin synthetic pathways in both platelets and vascular endothelium.

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PHS-6040

Project Description: Platelet-inhibitory drugs ibuprofen and sulfinpyrazone may have value in coronary disease. However, these drugs may block both platelet aggregation and synthesis of vasodilator prostaglandins. If platelets and blood vessels differ in sensitivity to ibuprofen and sulfinpyrazone, optimal doses might inhibit platelet aggregation but not interfere with synthesis of vasodilator prostaglandins. To seek such optimal doses we measured effects in dogs of ibuprofen, 0.1-3 mg/kg (n=7) or sulfinpyrazone, 0.3-100 mg/kg (n-9) i.v. on 1) vasodilation induced by the prostaglandins precursor arachidonic acid (AA) and 2) in vitro platelet aggregation. Mean rise in flow volume after 1 mg AA was 280 ml. Ibuprofen ≥1 mg/kg and sulfinpyrazone ≥10 mg/kg produced a mean reduction ≥60% ($p<.02$ for each) in this vasodilator response; Ibuprofen 0.1 mg/kg and sulfinpyrazone 0.3 mg/kg had no such effect. Flow rise after intracoronary PGE_2 30μg, was undiminished by ibuprofen or sulfinpyrazone. Ibuprofen 1 mg/kg and supfinpyrazone 10 mg/kg inhibited platelet aggregation induced by ADP, $2x\ 10^{-4}M$ ($p<.01$ for each). Ibuprofen 0.1 mg/kg and sulfinpyrazone 0.3 mg/kg did not alter platelet aggregation. Hence, ibuprofen and sulfinpyrazone doses inhibiting platelet function also produced comparable inhibition of AA-induced coronary vasodilation. Neither ibuprofen nor sulfinpyrazone appears to have an "optimal" dose that inhibits platelet aggregation without inhibiting synthesis of vasodilator prostaglandins.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01749-01 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Late Hemodynamic Evaluation of Porcine Heterografts in the Mitral Position

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

PI:	Lewis C. Lipson	Senior Investigator	CB	NHLBI
Other:	Kenneth M. Kent	Head, Cardiovascular Diagnosis	CB	NHLBI
	Douglas R. Rosing	Senior Investigator	CB	NHLBI
	Robert O. Bonow	Senior Investigator	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI
	Charles L. McIntosh	Senior Surgeon	SB	NHLBI
	Andrew G. Morrow	Chief, Surgery Branch	SB	NHLBI

COOPERATING UNITS (if any)
Surgery Branch, NHLBI, NIH.

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.05	.04	.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)

We have undertaken a study of the late hemodynamics of porcine heterografts and for mitral valve replacement. Preliminary data suggest that there is an appreciable incidence of valve deterioration manifested as prosthetic valve stenosis even in patients who do not show clinical evidence of valve dysfunction.

Project Description: The glutaraldehyde preserved porcine heterograft has become the valve of choice for mitral valve replacement. Report of ominous histological changes and occasional valve failures have raised questions of the long term durability of porcine heterografts in the mitral postiion. Since patients undergoing mitral valve replacement at the N.I.H. have routine cardiac catheterizations approximately six months postoperatively, we are able to assess changes in valve hemodynamics that have developed late postoperatively. Of 54 patients who underwent mitral valve replacement with porcine heterografts prior to October 1973, 28 are alive and potentially available for restudy. We propose to evaluate as many of these patients as possible with cardiac caterization.

To date 11 patients have been studied who have not had any clinical evidence suggestive of valve malfunction. Of these, four were found to have important increases in transvalve gradients (>5mmHg) and decreases in calculated valve areas (>0.5 cm^2). None of these patients has developed prosthetic mitral regurgitation. Two additional patients who had mitral valve replacements 98 and 61 months ago were studied because of clinical deterioration. Both were found to have evidence of severe prosthetic mitral stenosis, and one had developed severe mitral regurgitation.

These preliminary data suggest that the incidence of porcine heterograft deterioration after five years may be common. These findings, if confirmed by additional studies, may lead to the abandonment of porcine valves for foutine use in patients undergoing mitral valve replacement.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01750-01 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

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:	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 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	.4	.1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Verapamil has been administered to 55 patients with hypertrophic cardiomyopathy upon discharge from the National Heart, Lung, and Blood Institute. Thirty-eight patients have remained on the medication for a median follow-up time of 7 months. These patients have manifested an improvement in subjective symptomatic status as well as exercise capacity over this period of time.

PHS-6040
(Rev. 10-76)

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 (ex) capacity and subjective symptomatology in hospital in such patients. To assess the chronic effects of verapamil in HCM, 63 patients whose lifestyle was unacceptable despite propranolol therapy were begun on oral verapamil in hospital. Eight patients were not discharged on verapamil due to: side effects 4, non-compliance 2, symptomatic deterioration 1, death 1. The drug was stopped in 16/55 patients discharged on verapamil; in 13 because symptoms were unrelieved or recurred and in 3 because of drug side effects. One patient died while on the drug. Thirty-eight patients have remained on verapamil (1-20 mos; median = 7). Treadmill ex duration was evaluated in 18 of the 38. Long-term verapamil led to improved ex duration compared to in hospital a) control ($+63\pm14\%$; $p<.001$), b) propranolol ($+51\pm18\%$; $p<.025$), c) verapamil ($+36\pm8\%$, $p<.005$). Of the 63 patients started on verapamil in hospital, 26 (41%) have either improved their functional class by at least 1 grade and/or have increased their ex duration by 15%. Thus verapamil appears to offer a much needed new therapeutic approach t/o the treatment of HCM.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01751-01 CB

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Influence of Size of Risk Region on Incidence of Reperfusion Arrhythmia

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

P.I.:	Florence Sheehan	Clinical Associate	CB	NHLBI
Other:	Stephen E. Epstein	Chief, Cardiology Branch	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)

The size of the region at risk of infarction after coronary artery occlusion does not influence the incidence of ventricular fibrillation during reperfusion.

PHS-6040
(Rev. 10-76)

Project Description: Anesthetized foxhounds underwent occlusion of the circumflex coronary artery either proximal or distal to the first major obtuse marginal branch. After 20 minutes the occlusion was released and the artery reperfused. The incidence of arrhythmias during the first 2 1/2 minutes of reperfusion was monitored. The circumflex coronary artery was then permanently ligated and the chest closed. Three days later the dogs were sacrificed. To delineate the size of the region of myocardium at risk of infarction, the aorta was perfused with Evans blue and the circumflex with fluorescein in normal saline at equal pressures. The percentage risk region/total left ventricle was calculated. In 19 dogs who survived proximal occlusion, 9, or 47.4%, had ventricular fibrillation. In 11 dogs with distal occlusion, 5 or 45.5%, had ventricular fibrillation ($p>.05$). The mean risk region size was 35.82% of the left ventricle in dogs with ventricular fibrillation and 33.39% in those who survived reperfusion with sinus rhythm, ventricular premature beats, or transient ventricular tachycardia ($p>.05$). Thus, the location of the occlusion and the size of the resulting ischemic region did not influence the incidence of ventricular fibrillation. This is in contrast to arrhythmias occurring during coronary artery occlusion, which occur more frequently in occlusions resulting in large areas of ischemia.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01752-01 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Purification of Myosin Light Chain Phosphatase

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

PI:	Mary Pato	Fogarty Fellow	CB	NHLBI
Other:	Robert S. Adelstein	Head, Molecular Cardiology	CB	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Cardiology Branch

SECTION
Molecular Cardiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The enzyme, myosin light chain phosphatase is being purified using standard techniques including affinity column chromatography. Of particular interest is the specificity of the enzyme as well as those factors that regulate phosphatase activity.

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PHS-6040
(Rev. 10-76)

Project Description: The regulation of contraction-relaxation in smooth muscles requires the reversible phosphorylation of the 20,000 dalton myosin light chain. A full understanding of this mechanism necessitates the isolation and characterization of the enzyme, which dephosphorylates the light chain to effect relaxation. This project deals with the study of the properties of the enzyme myosin light chain phosphatase. Attempts have been made to isolate and purify the enzyme from turkey gizzards by $(NH_4)_2SO_4$ fractionation of a muscle extract followed by a series of chromatographic techniques including: gel filtration, ion exchange, Blue Sepharose CL-6B and an affinity column of myosin light chains. Two fractions of phosphatases of differing specificities have been isolated. One fraction is active towards phosphorylated histone and myosin light chains which the other fraction is active only toward the latter substrate. Differences in the activities of these two fractions in the presence of high (0.1M) Mg^{++} concentration have been observed. Purification of the enzymes to homogeneity and investigation of the specificities of these enzymes to other substrates particularly the phosphorylated form of myosin kinase and their activities in the presence of various effectors such as Ca^{++}, F^{-}, and small peptides, are currently being undertaken.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01753-01 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Cardiac Abnormality in Oculocraniosomatic Neuromuscular Disease

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

PI:	David. E. Schwartz	Clinical Associate	CB	NHLBI
Other:	John S. Gottdiener	Expert	CB	NHLBI
	Tulio F. Bertorini	Clinical Associate	MNB	NINCDS
	W. King Engel	Chief,Neuromuscular Diseases Sec.	MNB	NINCDS

COOPERATING UNITS (if any)
National Institute of Neurological and Communicative Disorders and Stroke, NIH.

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.03	.02	.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)

Oculocraniosomatic neuromuscular disease (ragged red fibers) has been associated with mitral valve prolapse and other cardiac abnormalitiies. Using echocardiography, 24 hour ambulatory ECG monitoring, and routine ECG, cardiac abnormality was noted in 7 (64%) of 11 patients studied. These included left anterior hemiblock, bifascicular block, supraventricular arrhythmia, and high grade ventricular ectopy. Mitral valve prolapse on echo was present in only 2/11 patients - neither of who had supporting auscultatory findings. Cardiac chamber dimensions and resting left ventricular function, determined by echocardiogram, were normal in all patients.

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Project Description: Oculocraniosomatic neuromuscular disease associated with ragged red fibers is a rare disorder in which a high frequency of mitral valve prolapse has been reported. To determine left ventricular function, chamber size, presence of mitral valve prolapse, and arrhythmia, we studied 11 patients, mean age 29 years, range 12-66 years, with echocardiography (echo), ECG and 24 hour ambulatory ECG monitoring. All patients had ptosis, opthalmoplegia, and characteristic ragged red fibers (caused by accumulation of abnormal mitochondria) on muscle biopsy. Other findings included: retinal pigmentary changes, short stature, partial hearing loss, and facial/ pharyngeal muscle weakness. Cardiac abnormalities were observed in 7 (64%) of 11 patients; ECG conduction abnormalities in 4/11 patients (2 bifasicular block and 2 left anterior descending hemoblock), supraventricular arrhythmias in 2/11 patients, and high grade ventricular ectopy in 3/11 patients. Mitral valve prolapse on echo was present in 2/11 patients - neither had systolic clicks. Cardiac chamber dimensions and left ventricular systolic function as manifested by fractional shortening were normal in all patients. Hence, cardiac involvement in this rare disorder is characterized by arrhythmias and conduction abnormalities with preservation of left ventricular function. Mitral valve prolapse, though present, is of minor significance.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01754-01 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Effect of Sulfinpyrazone and Naproxen on Myocardial Infarction Size

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

PI:	Roberto Bolli	Visiting Fellow	CB	NHLBI
Other:	Robert E. Goldstein	Senior Investigator	CB	NHLBI
	Nancy Davenport	Post-Doctoral Fellow	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	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:
.6	.5	.1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The effect of two platelet inhibitors, naproxen and sulfinpyrazone, on infarct size was studied in 38 open-chest dogs. Seventy-two hours after coronary ligation the heart was sliced and stained with triphenyl-tetrazolium chloride; infarct size measured by planimetry, was similar among control, naproxen-treated and sulfinpyrazone-treated animals. These results suggest that 1) platelet inhibition, per se, does not affect infarct size and 2) the infarct-sparing action of ibuprofen (a drug similar to naproxen) may be due to properties other than those common to naproxen.

PHS-6040
(Rev. 10-76)

Project Description: Sulfinpyrazone (SPZ), a platelet inhibitor, increases epicardial collateral flow. Naproxen (NAP), also a platelet inhibitor, is similar to the infarct-sparing drug ibuprofen (IB) but has greater lysosomal stabilizing activity. To evaluate the influence of these potentially beneficial agents on infarct size (IS), 38 open-chest dogs were randomly given i.v. either SPZ (n = 11, 30 mg/kg) NAP (n = 14, 30 mg/kg), or saline (n = 13) 10 min before, 3 and 6 hrs after ligation of the mid-left anterior descending coronary artery (LAD). SPZ and NAP markedly inhibited platelet aggregation. The dogs were sacrificed 72 hrs after occlusion. Myocardium at risk (MR), i.e., that supplied by the occluded artery, was identified by perfusing the distal LAD with saline and aortic root with Evan's Blue. Hearts were"bread-loafed" and stained with triphenyl-tetrazolium-chloride; IS and MR were measured by planimetry. IS/left ventricle was not significantly different among controls, SPZ and NAP groups (.28 ± .02, .30 ± .01, 28 ± .02, respectively) nor was IS/MR (.75 ± .03, .79 ± .03, .75 ± .03, respectively). Thus, neither SPZ nor NAP in platelet inhibitory doses altered IS. These results suggest that 1) platelet inhibition, per se, does not affect IS, 2) IB's infarct-sparing action may be due to properties other than those common to NAP.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01755-01 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Proteolysis in Dog Myocardium

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

PI:	Roberto Bolli	Visiting Scientist	CB NHLBI
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	Robert E. Goldstein	Senior Investigator	CB NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	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)

To verify the hypothesis that the rate of proteolysis is increased during ischemia, slices of ischemic and non-ischemic tissue from dog heart were incubated in either aerobic or anaerobic media. Tyrosine production, which is an index of the rate of protein breakdown, was measured by a spectrofluorimetric method. Preliminary results indicate that proteolysis is decreased by about 50% in both one and three-hour-ischemic tissue. Leupeptin, an inhibitor of thiol-proteases, decreased proteolysis by about 25% at 50μM. Furthermore, experiments are in progress to rule out the possibility that tyrosine production depends on the type of medium used.

PHS-6040
(Rev. 10-76)

Project Description: Biochemical, ultrastructural and immunohistological evidence suggest that lysosomal hydrolases may play a causal role in death of ischemic myocardial cells. To verify whether the rate of proteolysis is actually increased during ischemia, ligation of the left anterior descending coronary artery (LAD) was performed in dogs. After eigher 1 or 3 hours, the heart was excised, both the ischemic and nonischemic tissue sliced with a Stadie-Riggs microtome and the slices incubated for 3 hours at 37°C in either aerobic medium (Krebs-Ringer bicarbonate buffer - pH 7.40, saturated with O_2 95% - CO_2 5% and containing glucose 10mM, insulin 0.1 U/ml, chloramphenicol .04 mg, and cycloheximiole .5mM) or in anaerobic medium (differing from the former in that it was saturated with N_2 90% - CO_2 10%, had a lower $NaHCO_3$ content, pH 6.80 and lacked glucose and insulin). The amount of tyrosine produced by the slices was measured by a spectrofluorimetric method. Tyrosine is not degraded by cardiac tissue. In presence of cycloheximiole, which blocks protein synthesis, the production of this aminoacid is an index of the rate of protein breakdown. In some experiments, leupeptin, an inhibitor of thiol-proteases (including lysosomal cathepsin B) was added to the medium at concentration of 50, 200 and 500 μM. In experiments where the slices were preincubated for 50 min and then transferred to fresh medium, the production of tyrosine by nonischemic tissue in aerobic medium was .011 ± .001 (S.E.)nm/mg, 0.22 ± .003 and .035 ± .005 at 1, 2 and 3 hours of incubation (n=4). The corresponding values for 3-hour ischemic tissue incubated in anaerobic medium were 0, .008 ± .006 and .018 ± .005 (n=4). Leupeptin 500mM reduced proteolysis by 20 and 24%, respectively, after 3 hours of incubation. In experiments without preincubation, non-ischemic tissue in aerobic medium produced .074 ± .01 nm/mg at 3 hours of incubation (n=4) and the production was decreased by 26% and 15% by leupeptin 50 and 200 μM, respectively. Data on 3-hour ischemic tissue are still largely incomplete since the study is in progress. Partial results seem to indicate that proteolysis is decreased by about 50% in ischemic tissue, although it is possible that the observed difference is due to differences between aerobic and anaerobic medium rather than between ischemic and non-ischemic tissue. Further experiments are being done to verify this hypothesis.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01756-01 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Hypertrophic Cardiomyopathy in Friedreich's Ataxia

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

PI:	John S. Gottdiener	Expert	CB NHLBI
Other:	Rollin J. Hawley	Neurologist -V.A.Hospital, Washington, D.C.	
	Barry J. Maron	Senior Investigator	CB NHLBI
	Tulio F. Bertorini	Clinical Associate	MNB NINCDS
	W. King Engel	Chief, Neuromuscular Diseases Sec.	MNB NINCDS

COOPERATING UNITS (if any)

National Institute of Neurological and Communicative Disorders and Stroke,NIH.
Veterans Administration Hospital, Washington, D.C.

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.03	.02	.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)

Friedreich's ataxia has been associated with cardiac abnormalities similar to those seen in hypertrophic cardiomyopathy. To determine the presence of asymmetric septal hypertrophy and resting functional left ventricular outflow tract obstruction, 24 patients with Friedreich's ataxia and 14 asymptomatic first degree relatives were studied by echocardiography. The findings indicated that cardiac involvement in this disease is 1) characterized by concentric, and not asymmetric, left ventricular wall thickening, 2) is genetically linked with the neurologic abnormality, and 3) is not commonly associated with resting, functional left ventricular outflow tract obstruction.

217

Project Description: Features of idiopathic hypertrophic subaortic stenosis, including asymmetric septal hypertrophy and functional left ventricular outflow tract obstruction, have been described as characteristic cardiac findings in Friedreich's ataxia. In order to characterize cardiac anormality in Friedreich's ataxia, 24 such patients were studied by echocardiography (echo). Symmetric thickening of the the interventricular septum and the posterior left ventricular free wall was present in 13 (54%) of 24 patients. Increased left atrial dimension was present in 6 (46%) of these 13 points. Systolic anterior motion of the mitral valve was not present in any patient, suggesting the absence of resting left ventricular outflow tract obstruction. Left ventricular diastolic dimension was decreased in 8 patients, but was increased in one patient. This patient manifested decreased left ventricular fractional shortening. All other patients had normal fractional shortening consistent with intact systolic left ventricular function. Left ventricular wall thickness by echo was normal in 14 asymptomatic first degree relatives of patients with Friedreich's ataxia and thickened left ventricular walls. Hence, cardiac involvement in Friedreich's ataxia is: 1) characterized by concentric left ventricular wall thickening; 2) is genetically linked with the neurologic abnormality and 3) is not commonly associated with asymmetric septal hypertrophy or SAM.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01757-01 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Late Cardiac Effects of Therapeutic Mediastinal Irradiation

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

PI:	John S. Gottdiener	Expert	CB	NHLBI
Other:	Michael J. Katin	Clinical Associate	RO	NCI
	Jeffrey S. Borer	Senior Investigator	CB	NHLBI
	Stephen L. Bacharach	Physicist	NM	CC
	Michael V. Green	Chief, Applied Physics Sec.	NM	CC
	Lewis C. Lipson	Senior Investigator	CB	NHLBI

COOPERATING UNITS (if any)
Nuclear Medicine, Clinical Center, NIH.

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.1	.08	.02

CHECK 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 late cardiac effects of anterior mediastinal irradiation were assessed in 15 patients, 5-15 years after radiation therapy for Hodgkins disease. Diminished left ventricular ejection fraction (determined by ECG gated Tc^{99m} left ventricular cineangiography) with exercise was present in 5/15 (33%) of patients; average ejection fraction at exercise was also below normal for the group. Pericardial effusion on echocardiogram was present in 40% of patients. Asymptomatic impairment of systolic left ventricular function and pericardial effusion may be commonly present many years after mediastinal irradiation for Hodgkins disease.

219

PHS-6040
(Rev. 10-76)

Project Description: Therapeutic irradiation of the chest has been associated with pericardial, myocardial and coronary arterial injury. To assess the late effects of anterior mediastinal irradiation, 15 patients without prior cardiac disease were recalled for evaluation 5-15 years after radiation therapy for Hodgkins disease. Median age at evaluation was 34 years; range 25-59 years. ECG gated Tc^{99M} radionuclide left ventricular cineangiography at rest and exercise, and M-mode echocardiography (echo) were performed. Although ejection fraction (EF) at rest was depressed in only 2/15 (13%) radiation patients, the average for this group (0.52 ± 0.33 SEM) was below normal (0.57 ± 0.01, $p<0.05$). With maximum supine bicycle exercise EF was below normal in 5/15 (33%) patients. Average EF with exercise (0.58 ± 0.04) was below normal (0.71 ± 0.02, $p<0.05$). Regional wall motion abnormality was noted in one patient; a 33 year old woman with coronary artery disease. Pericardial effusion on echo was present in 6/15 (40%) patients. Asymptomatic restrictive cardiomyopathy was documented in another patient. We conclude that asymptomatic impairment of systolic left ventricular function, pericardial effusion and restrictive cardiomyopathy is evident many years following mediastinal irradiation for Hodgkins disease.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01758-01 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Cardiac and Autonomic Nervous System Changes of Self-Induced Starvation

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

PI:	John S. Gottdiener	Expert	CB	NHLBI
Other:	Walter Kaye	Staff Physician	ET	NIMH
	David Schwartz	Clinical Associate	CB	NHLBI
	Raymond Lake	Staff Physician	ET	NIMH
	Michael Ebert	Chief,Sec.on Exp.Therapeutics	ET	NIMH

COOPERATING UNITS (if any)
National Institute of Mental Health, NIH.

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.05	.04	.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)

Semi-starvation in anorexia nervosa and other conditions may be associated with sudden death and high output failure during rehabilitation. Abnormalities of heart rate, blood pressure and plasma catecholamines have been noted in anorexia nervosa patients. This study will evaluate cardiac and autonomic nervous system changes during nutritional rehabilitation of these individuals using non-invasive cardiac diagnostic techniques including echocardiography, exercise testing, 24-hour ambulatory ECG monitoring. Plasma norepinephrine at rest and exercise will be measured in parallel with these studies.

331

Project Description: Individuals with anorexia may suffer a mortality of 5-15%. This is the highest fatality rate for any psychiatric disease. The role of cardiac dysfunction or cardiac electrical instability in the death of patients with anorexia, if any, either during starvation or during re-feeding, is incompletely understood. In addition, anorexia provides an excellent clinical model for the study of uncomplicated protein-calorie deprivation. Despite the high incidence of sudden death in anorexia nervosa, the contribution of electrolyte abnormality, specifically metabolic alkalosis, has been uncertain and may contribute to sudden death. Of concern, sudden death has occurred during re-feeding of starved individuals who did not have anorexia nervosa, but who had undergone severe wright loss from voluntary adherence to a liquid protein hypocaloric diet. In addition, Keyes et al have observed high output failure to occur during nutritional rehabilitation of voluntary starvation. In addition to decreased cardiac dimensions, ventricular ectopy, hypotension, bradycardia and blunted heart rate and blood pressure response to exercise were noted in the starved state. In the presence of normal left ventricular function, these abnormalities may well indicate an aberration of autonomic function.

The purpose of this study is to examine biochemical and functional indices of autonomic nervous system function before and at intervals during nutritional rehabilitation of individuals who have undergone self-induced starvation in association with anorexia nervosa.

Publications: Gottdiener, JS, Gross, H.A., Henry, W.L., Borer, J.S., and Ebert, M.H.: Effects of self-induced starvation on cardiac size and function in anorexia nervosa. Circulation 58: 425-433, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01759-01 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Left Ventricular Dysfunction in Mitral Valve Prolapse

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

PI:	John S. Gottdiener	Expert	CB	NHLBI
Other:	Jeffrey S. Borer	Senior Investigator	CB	NHLBI
	Stephen L. Bacharach	Physicist	NM	CC
	Michael V. Green	Chief,Applied Physics Section	NM	CC
	Kenneth M. Kent	Head, Cardiovascular Diagnosis	CB	NHLBI
	Douglas R. Rosing	Senior Investigator	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

Nuclear Medicine Dept., NIH.

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.1	.08	.02

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Left ventricular function was studied in patients with mitral valve prolapse using Tc^{99m} gated equilibrium radionuclide cineangiography. Although ejection fraction at rest was normal for those subgroups of mitral valve prolapse patients with and without mitral regurgitation. The average ejection fraction with exercise for both groups was below normal. In patients with mitral prolapse, and no mitral regurgitation, there is a subgroup with diminished left ventricular functional reserve. In those patients with mitral regurgitation, decrease in left ventricular function may represent, in part, the effects of chronic volume overload.

223

Project Description: Abnormalities of left ventricular contraction have been noted in patients with mitral valve prolapse. In order to determine systolic left ventricular function in mitral valve prolapse, Tc^{99m} gated equilibrium radionuclide cineangiography was performed at rest and during maximal supine bicycle exercise in 25 patients with mitral prolapse who did not have coronary artery disease. Chest pain was noted by 15 patients, and arrhythmia was documented by ambulatory ECG monitoring and/or exercise electrocardiography in 10 patients. Ejection fraction at rest was normal in all patients (average 0.57 ± 0.02 SEM; normal 0.57 ± 0.01, NS). Although 13/19 patients with trivial or no mitral regurgitation increased ejection fraction during exercise, the average exercise ejection fraction for this group of 13 patients (0.60 ± 0.02) was below normal (0.71 ± 0.02, $p<0.001$). The remaining 6 patients without mitral regurgitation evidenced a decline in ejection fraction with an exercise ejection fraction <0.55. Of the 6 patients with mitral regurgitation, ejection fraction at rest was normal (average 0.56 ± 0.04) but declined with exercise in 4/6 patients (0.50 ± 0.07). Left ventricular end-diastolic dimension by echocardiogram was normal in all patients without mitral regurgitation. Chest pain and arrhythmia, though commonly present, did not predict left ventricular dysfunction. While many patients with mitral valve prolapse have normal left ventricular function, there is a subgroup with diminished left ventricular functional reserve. In those patients with mitral regurgitation, decrease in left ventricular function may represent, in part, the effects of chronic volume overload. In the absence of mitral regurgitation, left ventricular dysfunction is suggestive of a cardiomyopathic process.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01760-01 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Adriamycin Cardiotoxicity - Assessment by Radionuclide Cineangiography

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

PI:	John S. Gottdiener	Expert	CB	NHLBI
Other:	Douglas J. Mathisen	Clinical Associate	SB	NCI
	Jeffrey S. Borer	Senior Investigator	CB	NHLBI
	Robert O. Bonow	Senior Investigator	CB	NHLBI
	Charles E. Myers	Chief, Clinical Pharmacology	MB	NCI
	Stephen L. Bacharach	Physicist	NM	CC
	Michael V. Green	Chief, Applied Physics Sec.	NM	CC
	Steven A. Rosenberg	Chief of Surgery	SB	NCI

COOPERATING UNITS (if any)
National Cancer Institute, NIH.
Department of Nuclear Medicine, NIH.

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.2	.15	.05

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Radionuclide left ventricular cineangiography was used to study 19 asymptomatic patients who had previously received adriamycin 500 - 550 mg/m^2. Left ventricular dysfunction was present at median 4.8 months and at median 2.5 years following termination of adriamycin therapy. Adriamycin cardiotoxicity is evident in half of asymptomatic patients even at "safe" doses of drug, and may persist for several years without improvement.

PHS-6040
(Rev. 10-76)

Project Description: Adriamycin is an effective antineoplastic agent associated with dose dependent impairment of left ventricular function. However, it is uncertain whether changes in left ventricular function following adriamycin are reversible. Therefore, we used radionuclide cineangiography to study 19 asymptomatic patients without prior cardiac disease who had received adriamycin 500-550 mg/m^2 as adjuvant therapy for soft tissue sarcoma. Ejection fraction at rest and during exercise was determined in 10 patients (Group A) 1-12 months (median 4.8 months) and in 9 patients (Group B) 2-3 years (median 2.5 years) after completing adriamycin. Left ventricular ejection fraction at rest was abnormal (<45%) in 4 of 10 Group A patients (average = 47 $\pm$ 3%, normal average 57 $\pm$ 1%, $p<.005$), and was abnormal during exercise (<55%) in 5 of 10 patients (average = 53 $\pm$ 4%, normal average = 71 $\pm$ 1%, $p<.005$). Left ventricular dysfunction persisted in Group B patients as evidenced by decreased left ventricular ejection fraction at rest in 2 of 9 patients (average 51 $\pm$ 3%, $p<.01$) and during exercise in 6 of 9 patients (average 55 $\pm$ 3%, $p<.005$). There was no significant difference in left ventricular ejection fraction at rest or exercise between Group A and Group B patients. Hence, left ventricular dysfunction: 1) is evident following adriamycin in half of asymptomatic patients, even at "acceptable" cumulative doses of 550 mg/m^2,, and 2) may persist for several years without improvement.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01761-01 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

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
	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 Section	NM	CC
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

Nuclear Medicine Department, Clinical Center, NIH.

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.1	.08	.02

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Dilatation of coronary arterial obstructions by a balloon placed on a catheter is an effective way to restore functionally important and myocardial blood flow in selected patients with coronary artery disease.

PHS-6040
(Rev. 10-76)

Project Description: Angioplastic procedures on peripheral arteries with atherosclerosis was a technique developed 15 years ago by Dotter and Judkins. More recently, Gruntzig modified the original technique by using a polyvinyl chloride balloon fabricated at the end of the catheter so that the catheter with the balloon deflated could be positioned in the artery at the site of the atherosclerotic plaque and the balloon inflated to compress the plaque and enlarge the lumen of the vessel. In 1977 Dr. Gruntzig reported the results of using the balloon angioplastic procedure in the coronary arteries of four patients. Since the initial report, this angioplastic procedure has been attempted in approximately 210 patients world wide. In approximately 60% of the patients the balloon could be positioned in the coronary artery at the site of the atherosclerotic plaque and the balloon was inflated. In the remaining 40% of the patients the catheter could not be manipulated in such a way that the balloon was in the proper position. Approximately 50 of the patients in whom there was an initial increase in the diameter of the vessel angiographically, have undergone repeat angiographic studies 6 months to one year after the initial procedure. Ninety percent of the patients demonstrated either no change in the caliber of the vessel or an actual increase in the diameter of the vessel in the late study. The reasons for this late improvement are unknown, but it does appear that the angioplastic procedure results in more than just a transient enlargement of the vessel.

Two patients have undergone percutaneous transluminal coronary angioplasty at the N.I.H. In one patient a 70% obstruction of the left anterior descending coronary artery was reduced to a 30% obstruction. In that patient there was a decrease in the gradient across the obstruction from 65mmHg to 30mmHg. Table 1 lists the results of the graded exercise test, radionuclide cineangiogram and thallium perfusion scans in that patient before and after the angioplasty procedure. The patient was taking propranolol and long-acting nitrates prior to the procedure, but experienced incapacitating angina with less than usual activities. Now the patient is taking no medication, has returned to work and is engaging in unlimited physical activities. In the second patient there was a greater than 95% of obstruction of the right coronary artery. After the angioplastic procedures that was reduced to a 50% obstruction. The gradient across the obstruction fell from 65mmHg prior to the procedure to 30mmHg after the angioplastic procedure. In addition, there were left to right collaterals that disappeared immediately after the procedure. Table 1 lists the results of the exercise and radionuclide test done before and after the procedure. This patient was markedly symptomatic on beta blockers and long-acting nitrates prior to the procedure and is now asymptomatic on no medications and engaging in strenuous physical activities.

Table 1 on next page.

TABLE 1

	Graded Exercise Test			Radionuclide Angiogram		Thallium Perf.Scan	
	Time (min)	HR	ECG (ST segs)	Rest	Exercise	Rest	Exercise
MP pre :	3.5	138	5mm ↓	EF=58%(nl)	EF=53% ant.ap.hypo	nl	Ant. ap. hypoperf.
10 days post	22.5*	180	nl	EF=58%(nl)	EF=59% nl	nl	Ant.ap. hypoperf.
3 mo. post	22.5	180	nl	EF=58%(nl)	EF=62% nl	nl	nl
CG pre:	4.5'	110	2mm ↓	EF=56%(nl)	EF=57% inf. ap. hypokinesia	nl	Inf. ap. hypoperf.
10 days post	22.5'	160	nl	EF=53%(nl)	EF=60% nl	nl	nl

* End of test

nl = normal, ant = anterior, ap = apical, inf = inferior, hypoperf= hypoperfusion, EF = ejection fraction, segs = segments.

Thus, percutaneous transluminal coronary angioplasty appears to be an effective procedure for reducing the extent of angiographic obstruction. In addition, when successful, it affords symptomatic improvement and improvement in ventricular function during exercise and myocardial perfusion. Further studies will be necessary to determine the frequency of these salutory effects following the angioplastic procedure and just what the limitations of this technique will be. In summary, percutaneous transluminal coronary angioplasty appears to be an effective procedure in selected patients for reducing the degree of obstruction, improving symptoms, and improving the perfusion and the function of the myocardium.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01762-01 CB

PERIOD COVERED
March 1, 1979 to May 15, 1979

TITLE OF PROJECT (80 characters or less)
M-mode and 2-D Echocardiographic Assessment of Patients with Marfan Syndrome

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

PI:	Martin B. Leon	Clinical Associate	CB	NHLBI
Other:	Barry J. Maron	Senior Investigator	CB	NHLBI
	John S. Gottdiener	Expert	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:	PROFESSIONAL:	OTHER:
.01	.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)

Cardiac abnormalities, including aortic root dilatation and mitral valve prolapse account for the vast majority of deaths in the Marfan syndrome. In 11 patients with Marfan syndrome comparing M-mode echocardiography with two-dimensional sector-scanning, both techniques were comparable in assessing aortic root dimension. However, two-dimensional echocardiography affords added capacity to visualize aortic root morphology and the diagnosis of mitral valve prolapse is enhanced using two-dimensional echocardiography.

330

PHS-6040
(Rev. 10-76)

Project Description: Most patients with the Marfan syndrome have cardiac malformations including aortic root dilatation and mitral valve prolapse. M-mode echocardiography has been shown to demonstrate such abnormalities in many patients with the Marfan syndrome. To determine whether wide-angle, two-dimensional echo provides additional information compared with m-mode echo, 11 patients with Marfan syndrome, age 8-61 years, were studied. Analysis of transmural aortic dimension by two-dimensional echo from aortic and long axis views were comparable (r=0.97) and both agreed with m-mode aortic dimension (r=0.87). Increased maximal aortic dimension was detected in 10 of 11 patients by both techniques. In one patient the area of maximal aortic dimension could only be visualized with two-dimensional echo. Maximal aortic root dilatation was seen in the region of the sinus of Valsalva in all patients and two-dimensional echo accurately reflected angiographic aortic root contour in 5 patients studied. Mitral valve prolapse was present in 9 patients using two-dimensional echo, but only in 6 of those using m-mode echo. Hence, in patients with Marfan syndrome (1) two-dimensional and m-mode echo are comparable in assessing aortic root dimension; however, (2) two-dimensional echo affords improved ability to visualize the proximal aorta;(3) diagnosis of mitral valve prolapse is improved with two-dimensional echo.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01763-01 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Three Dimensional Echocardiographic Reconstruction of the Left Ventricle in Man

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

PI:	Thomas B. Stibolt	Senior Staff Fellow	LAS DCRT
Other:	Barry J. Maron	Senior Investigator	CB NHLBI
	John S. Gottdiener	Expert	CB NHLBI
	Stephen B. Leighton	Mech. Engineer	BEIB DRS

COOPERATING UNITS (if any)
Laboratory of Applied Studies, Div. of Computer Research and Technoligy, NIH.
Biomedical Engineering and Instrumentation Branch-Div. of Research Services, NIH.

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)

Three-dimensional reconstruction of the left ventricle may be accomplished utilizing sequential 2-dimensional short axis echocardiographic images. Such reconstruction may improve assessment of alterations in left ventricular structure and function in patients with cardiac disease.

PHS-6040
(Rev. 10-76)

Project Description: Cardiac diseases involving the left ventricle produce regional abnormalities in wall thickness and contractility which may be qualitatively defined by 2-dimensional echo. Optimally, an appreciation of the location and extent of such abnormalities requires three dimensioanl reconstruction of left ventricular structure. However, echo beam angulation from a single transducer location makes simultaneous imaging of corresponding portions of left ventricle in the same 2-dimensional short axis image impractical. Hence a technique was devised to permit accurate 3-dimensional reconstruction of left ventricle. Sequential short axis wide angle 2-dimensional echo images were obtained from a standard parasternal interspace at 2 degree intervals, from cardiac base to apex. A probe holder with integral protractor stabilized to the chest wall was used. Two-dimensional images were processed by a digital computer which corrected for aberrations in wall thickness using trigonometric formulae. Thus, it was possible to overcome inaccuracies introduced by beam angulation and reconstruct left ventricular structure as it would appear in truly parallel 2-dimensional images. Hence, 3-dimensional echo reconstruction of left ventricle from 2-dimensional short axis images is possible and may improve assessment of alterations in left ventricular structure and function in patients with cardiac disease.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01764-01 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Unusual Locations of Hypertrophy 2-D 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	Expert	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:	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)

Wide angle two-dimensional echo may identify unusually located areas of cardiac hypertrophy in patients with a family history of hypertrophic cardiomyopathy, but normal M-mode echocardiogram and abnormal ECG.

234

PHS-6040
(Rev. 10-76)

Project Description: M-Mode echocardiography has proved useful in the diagnosis of hypertrophic cardiomyopathy with asymmetric septal hypertrophy. However, relatives of patients with hypertrophic cardiomyopathy may exhibit normal M-mode echos even though their ECG is abnormal. To determine whether such relatives have hypertrophic cardiomyopathy unrecognized by M-mode echo, 20 such patients were studied using wide-angle 2-dimensional (2-D) echo to reconstruct the geometry of the entire left ventricular wall. Patients were 5-49 years old (mean 17); 17 were asymptomatic. Most common ECG abnormalities were Q waves, ST-T changes and right ventricular hypertrophy. M-mode echos showed septal-free wall ratio <1.3 and no septal hypertrophy. In 18 (90%) of 20 patients, 2-D echo showed localized areas of left ventricular wall hypertrophy. Such "lumps" involved regions of the heart through which the M-mode beam does not usually pass - i.e., the posterior septum (5 patients), anterior left ventricular free wall (6 patients), lateral left ventricular wall (6 patients) and the septum near the apex (1 patient). Hence: 1) M-mode echo may not identify hypertrophic cardiomyopathy in certain patients; 2) in such patients ECG may be a more sensitive indicator of hypertrophic cardiomyopathy than M-mode echo; 3) wide-angle 2-D echo is necessary to identify these unusually located areas of cardiac hypertrophy.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01765-01 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Etiology of Sudden Death in Athletes

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

PI:	Barry J. Maron	Senior Investigator	CB NHLBI
Other:	William C. Roberts	Chief, Pathology Branch	PB NHLBI
	Hugh A. McAllister	Armed Forces Institute of Pathol.	-Wash, D.C.
	Douglas R. Rosing	Senior Investigator	CB NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB NHLBI

COOPERATING UNITS (if any)

Pathology Branch, NIH, NHLBI.
Armed Forces Institute of Pathology, Washing, D.C.

LAB/BRANCH

Cardiology Branch

SECTION

Clinical Physiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.9	.8	.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)

Sudden death occurring in competitive athletes is usually due to structural congenital cardiovascular disease. The most common cause of such deaths in this series was hypertrophic cardiomyopathy. While the suspicion of cardiovascular disease is often raised during life in such athletes, the correct diagnosis is not often made clinically.

PHS-6040
(Rev. 10-76)

Project Description: Youthful competitive athletes epitomize the conditioned, healthy segment of society. However, such athletes may die unexpectedly. To determine the causes of such sudden deaths, comprehensive necropsy studies were performed in 29 competitive athletes. Ages ranged from 14-30 years (mean 20). Sudden death occurred during or just after severe exertion in 26. The most common sports participated in were football and basketball. Structural cardiovascular alterations were identified in 28 of 29 patients, with the most common being hypertrophic cardiomyopathy (14 patients). Other diseases included idiopathic concentric left ventricular hypertrophy (5 patients), anomalous origin of the left coronary artery from anterior sinus of Valsalva (3 patients), coronary artery disease (3 patients), ruptured aorta with the Marfan syndrome (2 patients) and coronary artery hypoplasia (1 patient). Cardiac disease had been suspected clinically in 7 but in only one was the clinical diagnosis correct. Hence: 1) Sudden death in competitive athletes is usually due to structural cardiovascular disease; 2) the most common cause of sudden death was hypertrophic cardiomyopathy, while coronary artery disease was relatively uncommon. Knowledge of the causes of sudden death may serve as a basis for designing screening programs for prospective identification of cardiovascular disease in athletic populations.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01766-01 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Specificity of Systolic Anterior Motion of the Anterior Mitral Leaflet (SAM)

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	Head, Cardiology Consultation	CB NHLBI
	Lowell W. Perry	Staff Cardiologist	Children's Hosp.Med.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:	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)

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. 10-76)

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). Ture 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 specificity of 98%. Of note, SAM was rarely fround 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: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01767-01 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

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	Head, Cardiology Consultation	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:	PROFESSIONAL:	OTHER:
.11	.1	.01

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

In patients with hypertrophic cardiomyopathy, 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.

240

Project Description: M-mode echo has shown asymmetric hypertrophy of the ventricular septum to be a consistent feature of hypertrophic cardiomyopathy. However, to better characterize distribution of hypertrophy throughout the left ventricular wall, 74 patients with hypertrophic cardiomyopathy were studied by wide angle 2-dimensional echo. Hypertrophy was confined to ventricular septum in only 20 patients, involved both ventricular septum and anterolateral left ventricular free wall in 43 patients and only left ventricular free wall in 11 patients. Posterior left ventricular wall was least hypertrophied. In 46 of the 74 patients hypertrophy was judged to be marked and diffuse, involving left ventricular free wall and/or ventricular septum; these patients were compared to 28 with mild, localized hypertrophy. Moderate-severe symptoms were more common with diffuse, (38/46, 83%) than with localized hypertrophy (6/28, 21%, $p < 0.001$). Left ventricular outflow obstruction was also more common with diffuse (29/46, 63%) than with localized hypertrophy (4/28, 14%, $p < 0.001$). Hence, 1) in patients with hypertrophic cardiomyopathy 2-dimensional echo shows a variety of patterns and greater distribution of hypertrophy than is appreciated by M-mode echo; 2) while hypertrophy is asymmetric, it often involves anterolateral left ventricle and usually is not limited to ventricular septum; 3) functional limitation and outflow obstruction is most common in those patients with marked, diffuse hypertrophy (i.e., greatest cardiac mass).

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01768-01 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Cellular Disorganization in LV Wall of Patients with Hypertrophic Cardiomyopahty

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	CC NHLBI
Other:	Thomas J. Anan	Guest Worker	CC NHLBI
	Noriko Sato	Guest Worker	CC NHLBI
	William C. 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:	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)

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.

242

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 section of ventricular septum, anterior and posterior left ventricular from 45 patients with hypertrophic cardiomyopathy and 85 patients with other cardiac diseases, using photographic enlargement and video planimetry. In patients with hypertrophic cardiomyopathy, ventricular septum disorganization was extensive (mean area disorganized 36 $\pm$ 4%). Disorganization was also marked in the LV free wall (anterior and posterior), mean 26 $\pm$ 4%, and was as extensive in anterior left ventricle (mean 32 $\pm$ 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 ventricular septum + left ventricular free wall disorganization (mean 43 $\pm$ 6%) was in 15 patients with hypertrophic cardiomyopathy ($\leq$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: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01769-01 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Nitroglycerin and Infarct Size

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

PI:	Florence Sheehan	Clinical Associate	CB	NHLBI
Other:	Robert E. Goldstein	Senior Investigator	CB	NHLBI
	Roberto Bolli	Visiting Fellow	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	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:
.6	.4	.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)

Nitroglycerin may only reduce the size of small myocardial infarctions in dogs without heart failure. The size of the region at risk of infarction may influence drug effect.

PHS-6040
(Rev. 10-76)

Project Description: We studied nitroglycerin (TNG)'s effect in reducing infarct weight in conscious dogs. Ten minutes after permanent left anterior descending coronary artery occlusion, ten dogs received nitroglycerin (450 mcg bolus IV then 300 mcg/min for 4 hours) and methoxamine as needed to maintain blood pressure and heart rate. No dogs were in failure. Ten control dogs received saline. Dogs were sacrificed 3 days later. Region at risk of infarction was delineated by simultaenously perfusing the aortic root with Evan's Blue and the distal left anterior descending artery with saline under equal pressures. Slices of stained hearts were incubated with tetrazolium to identify infarct (with histological confirmation). Total weight of left ventricle, region at risk and infarct were measured. TNG-treated dogs showed no difference from control in infarct weight (26.2 ± 5.9 g ± SE vs 27.7 ± 5.6), % risk region/left ventricle (36.0 ± 1.4 vs 37.9 ± 3.1) or % infarct/left ventricle (23.5 ± 5.2 vs 24.8 ± 4.9). In a subgroup with risk region/left ventricle by ≤35% nitroglycerin reduced infarct weight by 45% (8.8 ± 8.5 vs 15.9 ± 7.9) % infarct/left ventricle by 49% (7.1 ± 6.8% vs 13.8 ± 6.6), and % infarct/risk region by 41% (23.0 ± 22.0%, vs 38.9 ± 15.9). Due to small numbers of dogs, differences were not significant. In dogs with risk region/left ventricle >35% nitroglycerin had no effect. Thus, in dogs without overt heart failure, nitroglycerin may only reduce the size of small infarcts . These results suggest the size of the risk region may influence drug effect.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01770-01 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

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 F. Brensike	Coordinator	CB	NHLBI
Other:	Daniel J. Moriarty	Staff Associate	CB	NHLBI
	M. Myrianthopoulos	Dietitian	CB	NHLBI
	Beverly Rogers	Registered Nurse	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI
	Robert I. Levy	Director, NHLBI	OD	NHLBI
	Marian Fisher	Project Officer	BM	NHLBI

COOPERATING UNITS (if any)
Cardiology and Lipid Matabolism Branches of NHLBI.

LAB/BRANCH
Cardiology Branch

SECTION
NHLBI- Type II Coronary Intervention Study

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.8	.3	.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 randomized double-blinded study to determine whether lowering LDL cholesterol with diet and cholestyramine in patients with premature coronary artery disease and Type II hypercholesterolemia will retard the progression of coronary artery disease. Over one half the participants will have completed the study by October 1979 and it is anticipated that the study's conclusions will be available by 1981.

PHS-6040
(Rev. 10-76)

Project Description:

During the fiscal year 1979 several noteworthy events have occurred.

1. Data transfer of all basic data to the new Coordinating Center has been completed (additional data in family history, exercise tests, etc. are still being transferred). The quality and completeness of the data appear to be good.

2. A paper entitled "Coronary Calcifications and ECG Exercise Testing in the Detection of Coronary Artery Disease: Results from the National Heart, Lung, and Blood Institute's Type II Coronary Intervention Study" has been published in Circulation.

3. A baseline paper on coronary angiography reading methodology is in preparation.

4. The participants in the program have continued to be processed in an orderly fashion and over one half of the study participants will have completed the study by October 1, 1979.

5. No significant untoward side effects and no definitive answer concerning medication efficacy has been reported.

The next year should see continuing data collection on the remaining participants and increasing use of the data repository for data analysis and reporting.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01771-01 CB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Effects of Exercise Conditioning on Plasma High Density Lypoproteins

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

PI:	Lewis C. Lipson	Senior Investigator	CB NHLBI
Other:	Robert O. Bonow	Senior Investigator	CB NHLBI
	Ernst J. Schaefer	Staff Associate	MDB NHLBI
	H. Bryan Brewer	Chief,Molec.Dis.Branch	MDB NHLBI
	Frank T. Lindgren	Physician	Donner Laboratory, Univ.of Cal.,Berkeley

COOPERATING UNITS (if any)
Donner Laboratory, University of California, Berkeley.

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.1	.08	.02

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Physical conditioning has been thought to result in an increase in high density lipoproteins (HDL), a lipoprotein which has been associated with a lower incidence of coronary artery disease. We found that when body weight and diet were controlled, a six week program of exercise conditioning did not significantly alter HDL in young normal subjects.

PHS-6040
(Rev. 10-76)

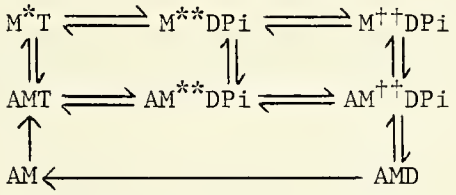
Project Description: Epidemiologic studies have demonstrated an inverse correlation between high density lipoprotein (HDL) cholesterol and the incidence of coronary artery disease. Although physically active individuals tend to have higher HDL levels than their sedentary peers, it has yet to be shown that physical activity by itself can raise HDL when other variables are maintained constant. We examined the effect of a six week exercise conditioning program on ten young normal subjects who were maintained on a constant composition, iso-weight diet. A training effect was documented by an increase in maximum oxygen consumption from 44 to 49 ml/min/kg and by a fall in heart rate at submaximal exercise from 120 to 109 beats/min. Total cholesterol was unchanged in the 4 subjects with <150 mg/dl prior to training. However, it decreased from 172 to 144 in those subjects whose cholesterol at start of study was >150. There was no significant change in triglyceride, very low density lipoproteins or low desnity lipoproteins. HDL cholesterol decreased insignificantly from 47 to 43 mg/dl, and total HDL and HDL subfractions measured by analytic ultracentrifugation were unchanged. Thus, under the conditions of this study in which diet and weight are controlled, exercise conditioning did not elevate HDL.

Publications: None

Annual Report of the
Laboratory of Cell Biology
National Heart, Lung, and Blood Institute
October 1, 1978 to September 30, 1979

The major activities of this Laboratory continue to be organized around the problems of: (1) muscle biochemistry; (2) cell motility; (3) microtubules; (4) electron transport. In addition, other problems under study include endocytosis and membrane flow in amoebae, attempts to characterize a high molecular weight DNA-synthesizing complex of Escherichia coli, the structure of fibrinogen, and the relation between protein disulfide bonds and protein conformation. Only the major features of selected individual reports are included in this summary.

(1) Muscle Biochemistry: Muscle contraction involves the interaction of actin thin filaments with myosin thick filaments. The "heads" of myosin molecules extend from the thick filaments and attach to the thin filaments. The energy released by the hydrolysis of ATP by myosin is converted into movement of the actin filaments relative to the myosin filaments. The nature of this energy transduction remains one of the central questions in muscle biochemistry. Previously, Dr. Eisenberg and his collaborators had proposed a new model for the molecular events derived from experimental data obtained with the soluble fragment of myosin, subfragment-1. In this model M stands for myosin; T for ATP and D for ADP.



This model differs from previous models proposed by Dr. Eisenberg and others in that myosin is always bound to actin and ATP is hydrolyzed by myosin in the actin-bound as well as the unbound form.

A thorough effort this year to determine by computer modelling whether a model simpler than that shown above could explain the experimental data has ruled out all other likely possibilities. In addition, new preliminary experimental evidence has given further support to the above model. The finding is that the initial burst of Pi release, which is faster than the steady state rate of hydrolysis of ATP, increases with increasing actin concentration, as predicted by the model, rather than decreasing, as would be expected from the alternative model originally proposed by Lymn and Taylor, or by modifications of it. These experiments will be continued in order to obtain more definitive evidence.

In any model for muscle contraction, the nature of the interaction of the two heads of myosin with F-actin is a central question. Model experiments are most simply performed with the single-headed myosin derivative, subfragment-1 and, with somewhat more difficulty, with the two-headed derivative,

heavy meromyosin. Previous studies in this laboratory have shown that, in the absence of nucleotide and at 22° and μ=0.22, heavy meromyosin binds to actin 600-fold more strongly than subfragment-1 with association constants of 3×10^9 M^{-1} and 5×10^6 M^{-1}, respectively. Under these conditions, the first head of the heavy meromyosin binds with an association constant of 10^6 M^{-1} (second-order reaction) and the second head with an association constant of about 10^3 (first-order reaction). The binding of the second head will be influenced both by the entropy of that head and the distortion necessary to introduce into heavy meromyosin for both heads to bind.

More detailed studies of the binding of subfragment-1 and both heads of heavy meromyosin have now been carried out under a variety of experimental conditions. In the presence of nucleotides, at different temperatures and at different ionic strengths, the binding of each of the heads of heavy meromyosin was affected to the same extent as the binding of subfragment-1. Also the binding constant for nucleotide to each of the heads of heavy meromyosin is the same as the binding constant for subfragment-1. These data indicate that, under all conditions of binding of myosin to actin, the two heads maintain a constant relationship.

The above, and other, experimental data, have been integrated into a modified cross-bridge model for muscle contraction in which the binding of ATP to the myosin-actin bridge leads not only to a dissociation of the actin and myosin but also to a change in the preferred angle of attachment of the cross-bridge from 45° to 90°. Thus, the cross-bridge could remain briefly attached at a much higher free energy, because it is now at 45° when it prefers to be at 90°, and then rapidly detach. This allows ATP to dissociate actomyosin with no loss in free energy.

(2) Cell Motility: The two proteins, actin and myosin, are also the major elements in the motility systems of all eukaryotic non-muscle systems. As for muscle contraction, the central problem is to understand the mechanism of the transduction of the chemical energy in ATP into movement. In contrast to muscle, however, the structural elements of the non-muscle motility systems are in a dynamic state. Specifically, non-muscle cells continually modulate the state of polymerization of actin and the interaction of actin filaments with each other. Dr. Korn and his collaborators have been studying these phenomena in two systems: the actively motile soil amoeba, Acanthamoeba castellanii, and the mammalian red blood cell.

A 14,000 dalton protein that regulates the polymerization of actin has been purified to homogeneity from Acanthamoeba. It is very similar, but significantly different, in its properties to a protein, profilin, previously isolated by others from spleen and platelets. It has now been found that profilin inhibits the first step of actin polymerization, the interaction of several monomers to form an actin "nucleus", but not the second step, the elongation of actin nuclei into long filaments. Acanthamoeba contains sufficient profilin to form a 1:1 complex with all of the non-polymerized actin in the cell (approximately 50% of the total actin) and, thus, this protein is probably the primary means by which the cell maintains a pool of non-polymerized actin.

All cells contain one or more proteins that cross-link filaments of F-actin to form networks or para-crystalline bundles. The shape of the red blood cell is believed to be maintained by a cytoskeleton consisting of actin and several other proteins, in particular spectrin which is a heterodimer of two proteins of 220,000 and 240,000 daltons. Others have shown the spectrin dimer can be reversibly converted to a tetramer. A number of different and incompatible models have been proposed for the interaction of actin and spectrin in erythrocytes. Recent work in this laboratory seems to have greatly clarified the situation. It has been found that spectrin dimer will bind to F-actin, as measured by co-sedimentation assays, but does not cross-link actin filaments. Spectrin tetramer, on the other hand, cross-links F-actin into gelled networks as measured by the large increase in viscosity. Contrary to reports of others, spectrin is equally active irrespective of its state of phosphorylation. Thus, spectrin dimer seems to have one binding site for F-actin monomers and spectrin tetramer two such sites. No interaction between spectrin dimer or spectrin tetramer and G-actin has been detected. Since the red blood cell does not seem to contain filaments of F-actin, the most likely structure for the erythrocyte cytoskeletal would be short oligomers of actin cross-linked by spectrin tetramers. Two other major proteins of the cytoskeleton probably interact with the spectrin component to stabilize the structure and to link it to the plasma membrane. This model is compatible with all available data and leads to several predictions on the properties of the spectrin/actin complex that can be isolated from erythrocytes. These predictions are now being tested and will be the major effort of the next year.

Previously, three myosin isoenzymes were isolated from Acanthamoeba castellanii: myosin II, a two-headed molecule of molecular weight about 400,000 and myosins IA and IB, both single-headed molecules of molecular weight about 180,000 but containing different polypeptide subunits. It had been shown by single-dimension peptide maps that the heavy chains of myosin IA and II must be products of different genes. It has now been shown, by the same technique, that the heavy chain of myosin IB is also a different gene product. All three enzymes are present in cultures derived from a single cell. Therefore, for the first time, a non-muscle cell has been shown to contain more than one myosin isoenzyme.

These results have been confirmed and extended by antibody studies. Rabbit anti-myosin II does not cross-react with myosin IA or myosin IB in radioimmunoassays or in direct precipitation tests using whole amoeba extract nor do anti-myosin IA or anti-myosin IB cross-react with myosin II. Myosin IA and IB do share antigenic determinants but they are still demonstrably different immunochemically. Each of the antibodies has also been shown to inactivate the actomyosin ATPase activity of its respective antigen. In addition to these results, the molecular weight of myosin IA has been more precisely determined as 150,000 by sucrose gradient centrifugation and sedimentation-equilibrium analysis. Also, it has not been possible to detect by immunochemical, or any other means, any protein of molecular weight greater than myosins IA and IB that might be a precursor of these unusually low molecular weight myosins.

Therefore, it seems increasingly likely not only that Acanthamoeba contains three myosin isoenzymes but that two of these are single-headed molecules of molecular weight about 150,000 that may not be able to form filaments. If these conclusions are correct, it will be necessary to propose models other than the generally accepted sliding filament model (based on muscle) for at least some actomyosin-dependent motile processes in the amoeba. The use of fluorescent labeled antibodies may enable the localization of the several myosins in the cell, and the ability of the antibodies to inhibit the enzymatic activity of their respective antigens may allow determination of the motile activities in which each of the myosins is involved.

(3) Microtubules: The major research efforts by Dr. Flavin and his collaborators involve the unique reaction by which a tyrosine residue is added to the C-terminal end of the α-chain of the tubulin dimer. Both the enzymology of the reaction and its possible biological significance are under study.

This year, a reproducible procedure was developed for obtaining 200-fold purification of tubulin-tyrosine ligase (the enzyme that adds the C-terminal tyrosine) from bovine brain. From the same source, they have obtained 500-fold purification of a specific detyrosylating enzyme that removes the C-terminal tyrosine from tyrosylated-tubulin. In contrast, however, to non-specific carboxypeptidase A, this enzyme does not remove any more amino acids from the detyrosylated α-chain. The enzyme is inhibited by GTP and colchicine but works equally well on polymerized and non-polymerized tubulin dimer as well as on isolated denatured single α-chains.

A potential clue to the biological function of this tyrosylation-detyrosylation was obtained last year when it was observed that membrane-associated tubulin from brain does not contain C-terminal tyrosine, although the purified tubulin could be tyrosylated, while cytoplasmic tubulin does. On the other hand, radioactive tyrosine was incorporated equally well into membrane-associated and cytoplasmic tubulin of cultured neuroblastoma cells. This same result has now been obtained for membrane and cytoplasmic tubulin of chick brain. Despite the fact that isolated membrane-associated chick brain tubulin contains no detectable C-terminal tyrosine, intracranially injected radioactive tyrosine is incorporated into membrane tubulin equally well as into cytoplasmic tubulin. This explanation of this paradox has not been found.

(4) Electron Transport: Dr. Hendler and his collaborators are continuing their studies of the mechanism of conversion of the energy of oxidation into metabolically usable energy. They have now developed more precise mathematical methods for analyzing the spectral data obtained by the novel electronic potentiometric titration technique described in last year's report. These procedures have led to the definition of several new components in the electron transport chain of Escherichia coli membranes and confirmed the discovery of 2-electron and 4-electron carriers. The potentiometric system has been further modified by addition of a microglass electrode to allow measurement of acid production as a function of the oxidizing potential of the system. It is hoped by this method to answer one of the major unsolved questions of the chemiosmotic hypothesis of Mitchell for coupling electron transport to energy production. Two acid-producing redox components have been tentatively iden-

tified. One of these seems to be an iron-sulfur protein with a redox potential similar to that of cytochrome oxidase. Preliminary data suggest that the protons generated by this component remain within the membrane (as proposed by Williams) rather than being used to form a pH gradient between the interior and exterior aqueous spaces (as proposed by Mitchell). It is planned to extend these studies with the bacterial membranes to studies of mammalian mitochondria to see if evidence for multielectron transfers and acid production can be obtained.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00401-13 LCB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Potentiometric studies of respiratory components of E. coli and rat liver mitochondria

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

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

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Division of Computer Research and Technology

B. Curve Fitting Analysis
Richard I. Shrager
Laboratory of Statistical and Mathematical Methodology
Division of Computer Research and Technology

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Section on Membrane Enzymology

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

New rigorous mathematical procedures were developed for isolating features of an optical spectrum which change during a potentiometric titration. Using these procedures and the system for automated electrode potentiometry, new redox components of the respiratory chain of E. coli have been found. The most important finding was that the cytochrome oxidase contains several components which are capable of scaling the 1-electron transport chain in two stages up to a 4-electron transfer for the complete reduction of dioxygen to water. Another important new finding is that a high potential iron-type redox component is present in the membranes and that this component is capable of liberating acid upon oxidation. Its redox potential is close to that of the cytochrome oxidase and the acid generation appears to accompany a 4-electron transfer process.

PHS-6040
(Rev. 10-76)

Project Description:

Methods of data analysis:

More precise methods for the mathematical resolution of spectral data have been developed. Two kinds of spectral analysis problems have been defined. In one case, the Gaussian absorption features are broad, easily defined, and overlapping. In this case the spectral region can be resolved into a series of individual Gaussian components having characteristic and fixed peak wavelengths, width to height ratios, and midpoint potentials. With these components plus an additional base-level component, the experimental curve can be reproduced over whole voltage range of interest. In the other case, a prominent and fairly narrow absorption peak stands out from the surface, but other less defined and smaller features are present nearby. In this case, trying to fit to Gaussians gives unsatisfactory results. However, the value of the second derivative at the peak wavelength isolates the major component from other nearby features. The value of the second derivative, which is strictly proportional to the size of the peak, is determined by fitting an inverted parabola through the peak and six other points closely and equally distributed on either side of the peak. The values thus obtained for either Gaussian heights or second derivatives are then individually analyzed as functions of voltage for Nernstian behavior to reveal the amounts of each, the midpoint potentials and the numbers of electrons transferred.

Analysis of the E. coli respiratory chain:

Using our electrodic system for potentiometry and the analytical methods described above, many repeated experiments were performed to define the respiratory chain of E. coli. Several new redox species have been defined. Assuming that all of the components are integrated in the respiratory chain in order of increasing affinity for electrons, we view the chain as a series of one electron trunks linking substrate to cytochrome oxidase. The cytochrome oxidase can coalesce first two 1-electron branches to one 2-electron branch and then two 2-electron branches to one 4-electron branch. This model offers a solution to the perplexing problem of how four 1-electron transporting chains can simultaneously reduce a molecule of O_2 to two molecules of water. Intermediate or partial states of reduction of dioxygen are of high energy and not considered likely intermediates.

Acid generating capability of the respiratory chain:

One of the greatest unsolved problems of the Mitchell Chemiosmotic hypothesis is the nature of the formation of the proton gradient. We have noticed and mentioned in the previous report that acid is generated during the oxidative titration of the E. coli cytochrome oxidase. In order to study this phenomenon more closely, the electrodic potentiometry system has been modified to incorporate a micro glass electrode which allows us to follow the generation of acid as a function of oxidizing potential. Using this system we have found that acid generation as a function of voltage displays Nernstian behavior. There appear to be two acid-producing redox components, one membrane-bound

and one soluble. The former corresponds to a 4-electron donor and appears to have the same midpoint potential as the 4-electron component of cytochrome oxidase. The latter behaves as a 2-electron donor and has a higher midpoint potential. The membrane-bound (i.e. 4-electron) component can be liberated from the membrane by ultrasonication leaving the cytochrome oxidase still membrane-bound. The spectral characteristics which correlate to acid production as a function of voltage are entirely different than those of cytochrome oxidase. The spectrum so far deduced for the acid producing component corresponds to those of typical iron sulfur proteins which are believed to play some important, but as yet undefined, role in the respiratory chain. One other fascinating observation of this system is that phosphate, a permeant anion, and FCCP, a proton ionophore, greatly enhance the acid generation process when membranes are used. Although Mitchell maintains that redox energy is converted to a pH gradient between the internal space and the external bulk phase, Williams believes that the energized proton always stays within the membrane and that the externalized protons represent leakage. The effects we have observed with phosphate and FCCP seem to be better explained by a Williams type model.

Proposed Course of Project: We plan to continue our studies of the acid-producing phenomenon in order to isolate the component involved and to investigate its possible role in the conversion of redox energy to metabolically usable forms. We also plan to study purified mammalian mitochondrial cytochrome oxidase to see if evidence for multielectron transfers can be demonstrated with this enzyme.

Publications:

Hendler, R.W.: Limitations on the use of and interpretation of data from the Aminco-Chance dual-wavelength split-beam recording spectraphotometer and related instruments. Anal. Biochem. 94: 450-464, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00402-07 LCB

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

DNA Synthesis in E. coli

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

PI: Richard W. Hendler,	Head, Sec. on Membrane Enzymology	LCB	NHLBI
Raymond Scharff	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, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.1	2.1	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Factor 'E,' isolated from E. coli and required for the stability of DNA-synthesizing complex, confers properties of the complex on free DNA polymerase I. The major quantity of natural complex in E. coli appears to be membrane-associated and new techniques for the isolation of this species are described. Factor 'E' is shown to be polymorphic. Techniques to manipulate interconversion of different forms are being explored to aid in the isolation of 'E'.

260

Project Description:

Background: E. coli contains a high molecular weight form of DNA polymerase I which utilizes a native DNA template in an ATP-stimulated DNA-synthesizing system. Previous studies indicate that the polymerase is complexed with exonuclease V (rec BC enzyme). The present report describes the further isolation of the complex and the distributions in the cell of the complex and a factor required for its stability.

Major Findings:

1. The previous report described many differences in the behaviors of active DNA-synthesizing complex and free DNA polymerase I in nondenaturing acrylamide gel electrophoresis. When free DNA polymerase is mixed with our recently discovered factor 'E', it acquires the properties of native complex in the gel system.

2. Purification of the complex is limited to procedures that do not require salt concentrations above ∿0.15 M because of its lability to salt. We have found that the complex can be adsorbed to hydroylapatite, ECTEOLA, and ATP-Sepharose and be eluted with low salt or ATP to achieve further purification.

3. Our earlier efforts of purification of the complex were directed to the 30 to 50% of total activity not sedimented at 20,000 x g. The most recent large scale preparation of E. coli, however, had only about 10% of non-sedimentable complex. Both the sedimentable and non-sedimentable forms of the complex are attached to DNA and we believe that the sedimentable form is further associated with membranes. Because of the distribution found in the new cell preparation, a completely new approach to isolation had to be developed. The procedure we are currently evolving uses DNAse to release the complex from the sedimentable fraction. The DNAse is removed by G-actin affinity chromatography and the free complex is rebound to DNA and passed through Bio-Gel A 50 m. The complex then emerges in the void fraction whereas most of the protein is included. The specific activity at this point is about fifty times that of the starting homogenate. Further purification, however, has been complicated by the fact that 'E' which is required to stabilize the free complex is no longer isolatable in the same manner as in the earlier preparations of E. coli. 'E' appears to be distributed in at least three forms. In one form it is sedimented at 20,000 g and not released by DNAse but is released by sonication. One soluble form is high molecular weight ($\sim 10^6$) and the other soluble form is a spectrum of much smaller sizes. The larger size can be obtained free of complex on Bio Gel A 50 m, but the smaller sizes are obtained with free complex. We are studying the inter-convertability of the two soluble classes of 'E' in order to force the formation of the larger size that can be obtained free of complex.

Proposed Course of Project: The purification to homogeneity of both complex and factor 'E' will be pursued, as well as the characterization of each and the study of the mode of action of 'E'.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00404-20 LCB

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Peptides liberated during plasmic and tryptic digestion of fibrinogen

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

Elemer Mihalyi	Research Chemist	LCB NHLBI
Masatsugu Kisaragi	Visiting Fellow	LCB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cell Biology

SECTION

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INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

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2	2	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Two classes of peptides are liberated by proteinases from fibrinogen: a) low molecular weight (LMW)-peptides, and b) high molecular weight (HMW)-peptides. Methods for the separation of these two classes have been worked out and the peptide fractions were characterized with respect to their amino acid compositions, peptide maps of the LMW-class, and optical properties related to secondary structure. The similarities and differences between the peptides liberated by plasmin or trypsin from human or bovine fibrinogen were established. Quantitative estimates were made of the peptide yields with respect to the native molecule and it was found that in all cases the segments removed by the enzymes are similar and seem to originate from the same regions of the molecule.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: About 1/3 of the fibrinogen molecule is liberated as high (HMW)- or low-molecular weight (LMW)-peptides during the fragmentation of proteolytic enzymes. Some of these peptides have biological activities. This, and the establishing of the origin and mechanism of formation of these peptides, makes their study desirable.

Methods Employed: Fractionation of the peptides by gel chromatography and precipitations; sodium dodecyl sulfate-polyacrylamide gel electrophoresis, amino acid analysis, optical rotation and circular dichroism measurements, estimation of digestibility of the peptide fractions.

Major Findings: Gel chromatography separates the peptides into the HMW- and LMW- classes. With plasmic digests these are well separated, with tryptic ones they overlap to some degree. The HMW-peptides are precipitated by 15% trichloroacetic acid or 60% saturated ammonium sulfate, whereas the LMW-peptides are not. The latter, on the other hand, are dialysable whereas the former are retained by cellophane membranes of 6000-8000 MW cut off point (Spectrapore 1). Either of these three methods can be used to prepare LMW- or HMW-peptides free of cross-contamination.

The HMW-peptides of plasmic digests have a molecular weight of approximately 22,000, whereas those of tryptic digests of 16,000. Trypsin digestion of the plasmic peptides yields the 16,000 MW fragment as a result of cleavage of a single bond. At a slower rate 3 more bonds are cleaved and two components of approximately 9000 and 6000 MW are formed. These are further degraded at an extremely slow rate.

Previous studies from other laboratories have demonstrated the α-chain origin of the HMW-peptide. Also its N-terminal sequence has been established. The sequence of the whole α-chain became recently available. All this localized the N-terminus of this fragment at residue 239 of the α-chain. Our amino acid and MW-data indicate that the plasmic fragment extends from Res. 239 to Res. 438 and the tryptic fragment from Res. 239 to Res. 406.

Optical rotation and circular dichroism studies do not show any secondary structure in either the 22,000 or the 16,000 MW fragment. However, amino acid analysis reveals 7 Lys and 11 Arg residues in the former and 4 Lys and 9 Arg residues in the latter (from human fibrinogen). From all these susceptible sites only one is cleaved at a fast rate in the plasmic peptide and 3 at a moderate rate in either the tryptic or plasmic peptides. Four Arg and one Lys residue is followed by a Pro residue and these are resistant to enzymatic attack. However, the remaining sites should be attacked readily. but they are not. This indicates that a compact structure of the chain should be present in this, in spite of the negative results of the optical methods (the latter would presumably show only the presence of α- or β-structures).

The low molecular weight peptides were resolved by peptide maps. With tryptic peptides the number of spots corresponds to the Lys and Arg content.

There are apparently no susceptible sites masked in those portions of the chains which yield these peptides. With plasmic peptides the number of spots is about 2/3 of the tryptic ones. This is in accord with the known more stringent specificity of plasmin as compared to trypsin.

Quantitative estimates of the peptide yields and their amino acid composition, with either trypsin or plasmin digestion of human or bovine fibrinogen indicated that in all these cases the segments removed by the enzymes are similar and seem to originate from the same regions of the molecule.

Significance to Biomedical Research: Since some of these peptides have immunological or pharmacological activities they may be of importance for the understanding of all the ramifications of the coagulation process.

Proposed Course of Project: This work is nearly completed. No continuation is planned.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00405-05 LCB

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Circular Dichroic Studies on Denatured Proteins

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

PI: Frederick H. White, Jr. Senior Investigator 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, Maryland 20205

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0.8	0.8	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

It is well established that development of conformational structure depends on amino acid sequence, although the exact relationship (which would make possible a precise prediction of native conformation from a knowledge of primary structure) remains unclear. It is widely accepted that native conformation develops from the randomly coiled chain of the denatured (or newly synthesized) protein by a process of nucleation, whereby a progressive folding, governed by thermodynamic factors, prevails until the native conformation is reached. As a consequence, the formation of S-S bonds would have nothing to do with folding but would occur in the latter stages to stabilize the native structure. However, it has been observed in this laboratory that native secondary structure is dependent on the existence of S-S bonds to the extent that the half reduced form assumes a circular dichroic behavior indistinguishable from that of the fully reduced protein. Further work is in progress to clarify the role of S-S bonds in chain folding.

PHS-6040
(Rev. 10-76)

Project Description

Objective: To examine conformational structures remaining in denatured proteins as they relate to the folding process.

Methods Employed

1. Methods developed in this laboratory have been used for partial and complete reduction of disulfide bonds.

2. Amino acid analyses to determine the carboxymethylcysteine content of reduced, alkylated samples have been performed on a Beckman Automatic Amino Acid Analyzer.

3. The Cary 60 Recording Spectropolarimeter has been employed for all studies of circular dichroism (CD).

4. Processing of CD data was accomplished with the PDP-10 computer, employing the "MLAB" system of curve fitting.

5. Gel filtration as a means of estimating the level of polymerization was carried out with Bio-Gel P-60 columns.

6. Enzymatic degradation was employed, followed by paper chromatography and electrophoresis ("fingerprinting") to examine the specificity of disulfide bond cleavage as well as the possibility of disulfide interchange during partial reduction.

Major Findings

Previous observations showed that reduction of lysozyme (to cleave two or more S-S bonds) results in a form that has a much lower helical content, but an increase in β sheet content (Annual Report No. Z01 HL 00405-04 LCB). It could therefore be postulated that this altered conformational form may be the preferred state in the absence of S-S bonds, and as such could be a precursor in the folding process.

However, many other workers have suggested that the reduced form is largely unfolded, yet retains a certain amount of native secondary structure. This interpretation would be compatible with a "nucleative" mechanism of chain folding, whereby the unfolded chain would develop native conformational characteristics under the influence of thermodynamic factors. The native S-S bonds would then form as a latter event in the folding process and would function mainly to stabilize the already folded structure. However, the findings of this laboratory, that an altered conformational form exists for both partially and fully reduced lysozymes, suggests an alternative possibility. Thus, an altered or "denatured" form of lysozyme exists at least in the early stages of S-S bond formation. When a critical number of native S-S bonds is reached, a transition may occur from the "denatured" to the native conformational structure. With this interpretation, S-S bonds would play a vital role in the folding process.

To differentiate between these hypotheses, it would be critical to examine the possibility that the observed "denatured" protein could give rise to a partially unfolded intermediate which could then fold in the hypothesized way by a nucleative mechanism. Thus a search has been undertaken for such an intermediate, possessing a native-like composition of helix, β sheet, and unordered structure, but without the greatly elevated β structure observed in this laboratory after reduction.

Accordingly, attempts have been made to repeat the work of Tamburro et al. (Int. J. Pept. Prot. Res. 2: 157 (1970)), as a result of which it was claimed that CD characteristics similar to those of native lysozyme were obtained on lowering the temperature to 2°C for reduced, carboxamidomethyl lysozyme. However, no temperature-dependent change could be repeated in this laboratory.

These workers, as well as Lee and Atassi (Biochem. 12: 2690 (1973)) have reported that 33-35% methanol causes a conformational shift of reduced lysozyme to native-like behavior. A shift was noted; however, curve fitting shows only small increases in both α helix and β structure, with no reversion to the native state.

The effects of pH have also been examined. Most of the work in this laboratory has been done at pH 3, at which level the solubility of reduced lysozyme is very high, with no tendency of the reduced form to reoxidize. Chain folding of this protein, however, is known to occur only at higher levels (pH 7-8). Thus, means have been elaborated for raising the pH (under an N_2 atmosphere) to make possible a pH study in this range. As a result, low helix and a high β structure were still in evidence. More importantly, there was no indication of reversion to native structure in the absence of S-S formation at the higher pH level.

The observed dependence of native secondary structure on S-S bonds supports the hypothesis that these bonds, formed at an early stage in the folding process, influence the subsequent course of native conformational development.

Significance to Biomedical Research

This effort helps to elucidate the events that occur during chain folding. Such information may contribute to understanding the relationship between primary structure and protein conformation sufficiently to permit reliable prediction of the conformation of the native molecule. This capability would be basic to the attack of many problems in biology and medicine, relating to the biological functions of proteins occurring in various conformational states.

Proposed Course of Project: Further experiments are contemplated to determine how and to what extent S-S bonds influence native conformational structure.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00409-09 LCB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

The Interaction of Actin and Myosin

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

PI:	Evan Eisenberg	Head, Section on Cellular Physiology	LCB	NHLBI
	Leonard Stein	Guest Worker	LCB	NHLBI
	Richard Schwarz	Staff Fellow	LCB	NHLBI
	P.B. Chock	Staff Investigator	LB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Section on Cellular Physiology

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.45	1.45	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)

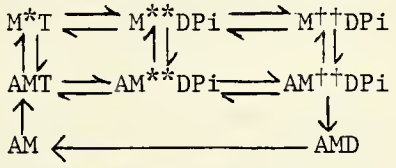
Computer modelling, steady state and pre-state kinetic studies on the interaction of actin, myosin subfragment-one and ATP were carried out in a continuing effort to elucidate the steps which occur when actin activates the myosin ATPase. Our results strongly suggest that myosin subfragment-one can hydrolyze ATP both when it is free in solution or bound to actin. Hence, there is no mandatory dissociation step in the actomyosin ATPase cycle.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The accepted mechanism of muscle contraction in vivo involves the interaction of the two proteins actin and myosin. These proteins which exist in vivo as filaments arranged in a highly ordered geometrical pattern interact by way of "cross-bridges" which extend from the myosin filaments. These myosin cross-bridges are thought to attach to actin monomers in the actin filament, and then, by means of an energy transduction mechanism, the energy stored in the ATP molecule is converted into motion of the actin filament relative to the myosin filament. The exact nature of this energy transduction mechanism is buried in the complexity of the specific interaction of the actin and myosin molecules. Hence the study of the actin-myosin interaction in vitro is of great importance to the understanding of muscle and will also have important implications for motily mechanisms as well as for other ATPase mechanisms.

On the basis of our work in the previous year we proposed a new model for the actin activated myosin subfragment-one ATPase in vitro:

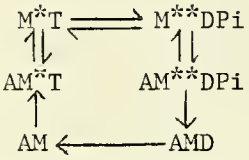


This model differs from previous models in that first, all S-1 states can bind to actin, that is, there is no true "refractory state" as originally described; and second, the model provides for the hydrolysis of ATP by S-1 while the mysoin is bound to actin. Our work this year was aimed at finding more evidence in support of our new model. First, detailed computer modelling was performed to see if a model, simpler than the one proposed above, could explain our data. Second, an attempt to measure the initial phosphate "burst" (rapid hydrolysis of ATP on the myosin surface) directly in the presence of actin is currently in progress in our laboratory.

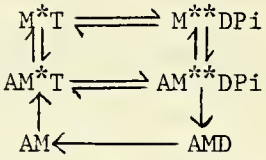
Methods and Findings

Detailed modelling was carried out by beginning with the simple Lymn-Taylor model (see below) and solving mathematically for the detailed predictions of this model in order to compare these predictions to our experimental data. The data used were: steady state behavior of the ATPase activity and steady-state and pre-steady-state turbidity behavior as a function of actin concentration. Based on the binding constants of $M^{*}\cdot T$ and $M^{**}\cdot D\cdot Pi$ to actin which we obtained from our turbidity data, we found that the Lymn-Taylor model predicted a considerable amount of inhibition of the actin activated S-1 ATPase at high actin concentration. We have never observed such inhibition. Other predictions of this model also were contradicted by our experimental results. Therefore we added further steps to the Lymn-Taylor model to determine if a simpler model than the one proposed above could fit our data. The next serious possibility is a modified form of the Lymn-Taylor model with

a step included to allow for the hydrolysis of ATP by S-1 bound to actin:



Lymn-Taylor model



Modified Lymn-Taylor model

This model does not predict inhibition of the actin activated myosin ATPase at high actin concentration, but it is still untenable because it does not fit the K_{app} given by the double reciprocal plot of ATPase vs actin concentration. This is because the K_{app} predicted by this model closely resembles the K for binding of S-1 to actin while experimentally the K for binding is four fold greater than K_{app}. Therefore we were able to rule out all models simpler than the model which we proposed which is given above. This mathematical analysis is presently in press as an appendix to the paper presenting our new data.

Next we proceeded to obtain direct evidence that the initial phosphate burst occurs whether the S-1 is dissociated from or bound to actin. That is, we began to search for stronger evidence that S-1 can hydrolyze ATP while remaining bound to actin. The mathematical modelling of the last section also predicted that, if the Lymn-Taylor model were correct, both the rate constant and the magnitude of the initial Pi burst would decrease with increasing actin concentration. Furthermore, the rate of the tryptophan fluorescence enhancement, used as a measure of the initial Pi burst in the absence of actin, should also decrease in the presence of actin according to the Lymn-Taylor model. In contrast, our model predicts that the rate of the initial Pi burst will increase with increasing actin concentration. Our model predicts that the magnitude of the initial Pi burst will decrease slightly but much less than the decrease predicted by the Lymn-Taylor model. We are now directly measuring the rate and magnitude of the initial Pi burst using the three syringe quench flow apparatus. We are also measuring the rate of the fluorescence enhancement using the two syringe stopped flow apparatus. Our preliminary results are consistent with our model but not with the Lymn-Taylor model.

Proposed Course of Project: During the coming year we will continue to investigate whether the S-1 ATPase occurs at the same rate when the S-1 is free or bound to actin. The evidence we are now collecting regarding the initial phosphate burst may rule out the Lymn-Taylor model as originally stated.

Another project we are now beginning is a detailed study of the nature of the binding of S-1 to actin in the presence of ATP. In particular we will study the ionic strength and temperature dependence of this binding. This will give us a clearer understanding of the physiological importance of this binding.

Finally, we are beginning a study where the "roles" of S-1 and actin are reversed - - that is, actin is at low concentration and S-1 is at high concentration, so that in effect actin is acting as the "enzyme". Eisenberg and Kielley previously used this method to determine the amount of S-1 bound to actin in the presence of ATP. Thus determination of the ATPase rate per actin monomer at high S-1 concentration as compared to the rate per S-1 at high actin concentration should provide an alternative method of determining the amount of S-1 bound to actin in the presence of ATP. We will, therefore, be able to use this method to test the validity of our turbidity experiments.

Publications

Chock, S.P. and Eisenberg, E.: The mechanism of the skeletal muscle myosin ATPase I. Identity of the myosin active sites. J. Biol. Chem. 254: 3229-3235, 1979.

Chock, S.P., Chock, P.B., and Eisenberg, E.: The mechanism of the skeletal muscle myosin ATPase II. Relationship between the fluorescence enhancement induced by ATP and the initial Pi burst. J. Biol. Chem. 254: 3236-3243, 1979.

Chock, S.P.: The mechanism of the skeletal muscle myosin ATPase III. Relationship of the H^+ release and the protein absorbance change induced by ATP to the initial Pi burst. J. Biol. Chem. 254: 3244-3248, 1979.

Stein, L.A., Schwarz, R.P., Chock, P.B., and Eisenberg, E.: The mechanism of the actomyosin ATPase: evidence that ATP hydrolysis can occur without dissociation of the actomyosin complex. Biochemistry in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00411-04 LCB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Reconstitution of E. coli succinoxidase system from sub-components

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

Richard W. Hendler	Head, Sec. on Membrane Enzymology	LCB	NHLBI
T.L. Parasad Reddy	Visiting Fellow	LCB	NHLBI
Oruganti H. Setty	Visiting Fellow	LCB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
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SECTION
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INSTITUTE AND LOCATION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.3	0

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Various methods to obtain quantitative information of the degree of asymmetry of native and reconstituted respiring membranes were explored. In oxygen pulse-induced pH jumps, the measured dH^+/dO ratio for spheroplasts was three with D-lactate and four with succinate as energy sources. With reconstituted succinoxidase vesicles in the presence of FCCP, dH^+/dO was about 0.7. The FCCP seemed to be required for H^+ transport. The development of a membrane potential in the reconstituted vesicles was indicated by the necessity for having valinomycin and K^+ present to allow proton ejection and by flow dialysis using valinomycin and ^{86}Rb.

PHS-6040
(Rev. 10-76)

Major Findings:

Various methods to obtain quantitative information of the degree of asymetry of the starting and reconstituted membranes were explored. All methods were based on the determination of vectorial movements and distributions of protons and other charged molecules. A filtration method to separate vesicles from medium was first tried. Internal volumes for energized and de-energized spheroplasts were determined by a centrifugal method using [^{14}C]-inulin and [^{3}H]-H_2O distributions. Energized spheroplasts had about 1.3 µl per mg protein and de-energized about 2 µl. The external water space of the pellet was about 13 µl. The measurement of a pH gradient across the spheroplast membrane based on [^{14}C]-acetic acid or [^{14}C]-DMO (a weak organic acid) distributions did not work because the internal pool was not stable to the filtration and washing procedures. In the previous report, acidification of the medium in response to a burst of respiration caused by injecting oxygenated buffer to an anaerobic suspension of reconstituted vesicles in the presence of D-lactate was cited as qualitative evidence of asymmetry. We have refined the system by introducing an oxygen electrode, determining the relaxation constant for the electrode and the leak rate for loss of injected oxygen to the anaerobic gas phase. An equation incorporating these terms was developed by Richard I. Shrager, DCRT. With this system we could measure simultaneously the rates of proton ejection and oxygen uptake. The experimental values obtained for spheroplasts were equal to the highest yet reported for intact E. coli., namely three protons ejected per atom of oxygen consumed using D-lactate and four using succinate as energy source. Further evidence for the integrity of the system was the necessity of providing valinomycin and K^+ to relax the membrane potential that prevents proton extrusion and the observations that proton ejection did not follow the injection of anaerobic buffer or of oxygenated buffer in the presence of uncoupler, FCCP. A very interesting finding was that DNAse treatment normally used in the preparation of the spheroplast suspention destroyed about 90% of the proton ejection-capability and eliminated the effects caused by valinomycin and uncoupler.

Succinoxidase vesicles reconstituted from crude cytochrome oxidase and crude succinate dehydrogenase do vectorially eject protons during an oxygen-induced pulse of respiration. Furthermore, the vesicles do develop a significant membrane potential as evidenced by the fact that valinomycin and K^+ must be present to allow proton ejection. However, the rate of proton ejection relative to oxygen uptake (dH^+/dO) is only about 0.3. This ratio can be raised to about 0.7 by addition of the proton ionophore FCCP, suggesting that the reconstituted vesicles may be exceedingly impermeable to protons. Possible reasons why the dH^+/dO rate is low in the reconstituted vesicels are:

1. The rapid removal of deoxycholate (DOC) during reconstitution causes a too rapid "crystallization" of membranes at the cost of proper spatial orientation.

2. The crude system retains an appreciable amount of DOC which may inhibit vectorial proton ejection.

3. A factor involved in proton ejection is being lost during the dissolution and reconstitution of the membrane.

A flow dialysis system was set up using valinomycin and ^{86}Rb to measure membrane potential and [^{14}C]-acetic acid to measure a pH gradient. In spheroplasts, a membrane potential could be established with D-lactate or succinate as energy sources, but no pH gradient was seen. These experiments were done with the usual spheroplast suspension that had been exposed to DNAse.

In view of the deleterious effect of DNAse exposure in the pulsed respiration-induced pH jump experiments described above, it will be important to repeat the flow dialysis experiments with spheroplasts not treated with DNAse. A preparation of crude succinoxidase vesicles did appear to develop a membrane potential while oxidizing succinate.

Proposed Course of Project: Efforts will be made to improve the energy transducing capabilities of reconstituted vesicles. These efforts will include the use of purified succinate dehydrogenase and cytochrome oxidase, the slow removal of deoxycholate during reconstitution and the removal of residual deoxycholate from reformed vesicles.

Publications:

Reddy, T.L.P. and Hendler, R.W.: Reconstitution of E. coli succinoxidase from soluble components. J. Biol. Chem. 253: 7972-7979, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 00413-03 LCB

PERIOD COVERED
October 1, 1978 to September 30, 1979

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

Lois Greene	Staff Fellow	LCB NHLBI
Evan Eisenberg	Head, Sec. on Cellular Physiology	LCB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Section on Cellular Physiology

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 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) The binding of heavy meromyosin (HMM), a soluble two-headed fragment of myosin, to F-actin was examined under a variety of conditions to determine the interaction occurring between the HMM heads in binding to F-actin. We find that the HMM molecules bind independently along the F-actin filament with a stoichiometry of one HMM/2 F-actin monomers in the presence of AMP-PNP or ADP, the same stoichiometry observed in the absence of nucleotide. In the absence of nucleotide (μ=0.22M, 22°,) HMM binds to F-actin about 600-fold stronger than the single-headed myosin fragment, subfragment-one (S-1), whereas there is only a 2-fold difference between the binding of HMM and S-1 to actin in the presence of saturating concentrations of AMP-PMP. These data, as well as results obtained at varied ionic strength and temperature show that there is a fixed relationship between the binding of S-1 to actin and the binding of each of the HMM heads to actin, i.e. the binding of each of the HMM heads to actin is always affected to the same extent as the binding of S-1 to actin. This shows there is a constant ratio between the association constants for the binding of the first and second heads of HMM to F-actin, suggesting that the amount of distortion which occurs in the HMM molecule when the second HMM head binds to F-actin remains the same under all experimental conditions.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The binding of the soluble subfragments of myosin to F-actin was studied under varied experimental conditions to determine the interaction occurring between the two heads of myosin when they both bind to F-actin. It was previously shown that the binding constant of heavy meromyosin (HMM), a two-headed fragment of myosin, to F-actin is 3×10^9 M^{-1} at μ=0.22M, 22°, while under the same conditions subfragment-one (S-1), a single headed fragment of myosin, binds to actin 600-fold weaker. To analyze the interaction between the two HMM heads when they both bind to F-actin, the binding of HMM can be treated as a two-step process. The first head of HMM binds to actin in a second-order reaction with an association constant similar to that found for S-1 ($K \approx 5 \times 10^6$ M^{-1}), following which the second head of HMM binds in a first-order reaction with $K \simeq 10^3$.

The strength of binding of the second head relative to the first head depends on the freedom of motion of the second HMM head before it binds to F-actin, as well as on the distortion which occurs in the HMM molecule when both heads are bound. In the present study, we investigated the effect of various factors, which are known to strongly affect the binding of S-1 to F-actin, on the binding of HMM to F-actin. An understanding of the interaction between the two HMM heads on actin, both in the presence and absence of nucleotide, should help us to understand how the two myosin heads interact with F-actin in vivo.

Methods Employed and Major Findings

The binding of HMM and S-1 to F-actin was examined under a variety of conditions: different ionic strengths, temperatures, and in the presence and absence of nucleotide. These studies were performed by mixing [^{14}C]-iodoacetamide modified fragments (HMM or S-1) and F-actin. After centrifuging the F-actin fragment complex, the concentration of fragment which remained in the supernatant was measured to determine the extent of binding under the different conditions. Similarly, experiments were performed in which there was a competition between HMM and S-1 for sites on F-actin to determine the actin-HMM association constant in the absence of nucleotide.

The binding of actin to HMM was first examined in the presence of nucleotide (ADP or AMP-PNP). There is a reduction in the actin-HMM association constant in the presence of AMP-PNP or ADP so that the binding of the HMM·nucleotide complex to F-actin can be measured directly. This experiment showed that the HMM molecules bind independently along the F-actin filament. The stoichiometry of binding is one HMM per two F-actin monomers in the presence of saturating concentrations of AMP-PNP or ADP, the same stoichiometry observed in the absence of nucleotide.

From competition studies, we previously found that HMM binds 600-fold stronger to actin than does S-1 in the absence of nucleotide at μ=22M, 22° i.e. S-1 and HMM bind to actin with association constants of 5×10^6 M^{-1} and 3×10^9 M^{-1}, respectively. In the presence of saturating concentrations of

AMP-PNP, we find that there is only a 2-fold difference between the actin-S-1 ($K=2 \times 10^4\ M^{-1}$) and actin-HMM ($K=4 \times 10^4$) association constants. Similarly, ADP weakened both the actin-S-1 and actin-HMM association constants, but to a lesser extent than AMP-PNP. Further studies at different ionic strengths and temperatures showed that all changes in experimental conditions including the binding of nucleotide to HMM affect the binding of each of the HMM heads to actin to the same extent as the binding of S-1 to actin. In agreement with these results, nucleotide binding studies showed that each HMM head binds nucleotide with a binding constant similar to that found for S-1, both in the presence and absence of actin.

These results strongly suggest that under a wide variety of conditions whenever the binding of the first HMM head to actin is changed, the binding of the second head is changed proportionally. Therefore, there is always a constant ratio between the binding constants of the first and second HMM heads to F-actin. This, in turn, suggests that the amount of distortion which occurs in the HMM molecule when the second HMM head binds to F-actin remains the same under all conditions.

Significance to Biomedical Research

The knowledge of the interaction of myosin with actin both in the presence and absence of nucleotide is fundamental in understanding the process of muscle contraction. Since both the attached and detached states of the actin-myosin complex were studied, this helps to elucidate the basic mechanism of the cross-bridge cycle. In addition, this research applies to events that occur during cell movement involving the contractile proteins actin and myosin.

Proposed Course of Research

The plan for this project is to continue investigating the interaction of actin and HMM in the presence of nucleotide to understand in detail the formation of the acto·HMM·nucleotide ternary complex. In addition, the mechanism of relaxation of muscle contration by the troponin-tropomyosin complex will be studied by examining the effect of the regulatory complex on the formation of the acto·S-1·nucleotide ternary complex. Another aspect of this research will be to compare the ability of HMM and myosin to bind F-actin both at high ionic strength where the myosin is soluble and at low ionic strength where the myosin aggregates.

Publications

Greene, L.E. and Eisenberg, E.: Dissociation of the actin-subfragment-one complex by adenylyl imidodiphosphate, ADP, and PPi. J. Biol. Chem. in press.

Greene, L.E. and Eisenberg, E.: The binding of heavy meromyosin to F-actin. J. Biol. Chem. in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00414-02 LCB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Structural and Functional Relationships in Concanavalin A

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

PI: Frederick H. White, Jr. Senior Investigator 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, Maryland 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)

Concanavalin A (con A) is one of several lectins which have been found to stimulate mitosis, in addition to causing agglutination of cells. This protein is the most extensively studied of the group, and X-ray studies in other laboratories have already established its three dimensional structure. Its biological effects appear to arise entirely from its ability to bind saccharides.

Attempts are being made, with the aid of circular dichroism and other optical methods, to obtain information on structural modifications within the con A molecule resulting from a variety of reactions, and to relate these changes to modifications in biological activity, as part of an investigation of mitogenic effects as a long range goal.

PHS-6040
(Rev. 10-76)

Project Description

Objective: To explore structural and functional relationships in lectins (with emphasis on concanavalin A (con A)) as an approach to the study of mitogenesis.

Methods Employed

1. Published methods are employed for the complexing of con A with saccharides and polysaccharides for the purpose of examining concommitant structural changes.

2. Modifications of con A structure by published chemical and enzymatic methods will be performed, as well as methods for removal and addition of metal ions essential for activity.

3. Standard chromatographic and electrophoretic methods are used for the isolation of derivatized forms of con A.

4. Studies of circular dichroic (CD) behavior are carried out with a Cary 60 Recording Spectropolarimeter.

5. The CD data are processed by a computer curve-fitting system ("MLAB") in the PDP-10 computer. The method gives estimates of fractions of chain length as α-helix, β sheet, and residual structure.

Major Findings

Essential for meaningful estimates of structural fractions is the choice of basis spectra that most nearly represent the CD behavior of each structural moiety within the protein. Thus, attention has centered around continued development of CD curve fitting for assessing small structural changes in conformational structure, especially of con A. An "additive" principle has been explored. Thus, basis spectra have been employed, derived from two sources:

1) experimental observation of CD behavior of polypeptides, and

2) calculation from structural fractions known to exist in the native protein from X-ray diffraction studies.

These spectra have been used in various combinations in attempts to improve upon exising published basis spectra for curve fitting. As a result, a combination of six basis spectra curves (two each for helix, β sheet, and residual structure) has been found to give approximations to the known structural fractions for eight out of eleven native proteins examined (including con A and lactate dehydrogenase, neither of which has ever been successfully curve-fitted by published basis spectra).

Significance to Biomedical Research

An understanding of mitosis is basic to the attack of many problems in biology and medicine. The present approach is aimed at studying some of the factors involved in initiating the process. Since attention has been focussed on the effects of lectins, small structural changes in these proteins on addition of cellular surface material (polysaccharide) have become critical to examine. Thus improvements in existing CD techniques have been sought, which could have significance for a wide variety of macromolecular structural changes related to biology and medicine.

Proposed Course

The investigation of CD curve-fitting methods will be continued, with the object of applying them to the study of small conformational changes in con A, associated with binding to the cell surface.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00415-01 LCB

PERIOD COVERED

October 1, 1978 to September 30, 1979

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

PI:	Evan Eisenberg,	Head, Sec. on Cellular Physiology	LCB NHLBI
	Lois Greene	Staff Fellow	LCB NHLBI
	Terrell Hill,	Head, Sec. on Theoretical Molecular Biology	LMB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Section on Cellular Physiology

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.6	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 new cross-bridge model has been developed based on the concept that the binding of ATP to the myosin-bridge-actin complex not only weakens the binding of actin to myosin but also increases the preferred angle of the attached cross-bridge from 45° to 90°. This concept 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 myosin does not have to dissociate from actin each time an ATP molecule is hydrolyzed. However, when the muscle is moving detachment of the cross-bridge does occur with no loss of free energy. This new model is therefore consistent with recent biochemical evidence suggesting that ATP hydrolysis can occur without mandatory dissociation of the actomyosin complex.

PHS-6040
(Rev. 10-76)

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. This kinetic model differs from our previous kinetic model in two major respects. First, ATP does not cause complete dissociation of the acto-S-1 complex; the refractory state is able to bind to actin at high actin concentration. Second, ATP hydrolysis can occur without ATP causing dissociation of the acto-S-1 complex. Third, a large free energy change occurs when ATP binds to actomyosin. In the present study we developed a new qualitative model of cross-bridge action in muscle based on this new kinetic model. We have also begun a theoretical study of the nature of the two headed binding of HMM to actin based on our experimental data with ATP analogues.

Methods Employed and Major Findings: The major assumption of our new cross-bridge model is that the conformational change associated with the binding of ATP to actomyosin not only weakens the binding of actin but also changes the preferred angle of the attached cross-bridge from 45° to 90°. This allows ATP to dissociate the actomyosin link when the muscle is in motion without any loss in free energy occurring. Of course, in this model the cross-bridge at 45° does not actually change its angle back to 90° when ATP binds. Although the cross-bridge in state A·M·ATP would prefer being at 90° at its minimum free energy, its elastic nature allows it to briefly remain attached at 45° at a much higher free energy because it is elastically distorted. It then rapidly detaches because the unattached and attached cross-bridges are in very rapid equilibrium. Thus in this new cross-bridge model we are combining the biochemical finding of rapid equilibria between states with the physiological concept of cross-bridge elasticity to explain cross-bridge behavior in muscle. At the same time the model offers a possible explanation for the action of ATP analogues both in vivo and in vitro. AMP-PNP and PPi seem to cause muscle fibers to lengthen rather than shorten. In vitro they dissociate actomyosin but not as well as ATP; they also bind to myosin much more weakly than ATP. Since in our model ATP changes the preferred angle of the cross-bridge from 45° to 90°, it seems reasonable that ATP analogues would also increase the preferred angle of the cross-bridge. However, just as they bind to myosin much more weakly than ATP, so too, it seems reasonable that they would increase the preferred angle of the cross-bridge less than ATP, e.g. from 45° to 55°. Such a small increase in preferred angle could explain the ability of ATP analogues to lengthen muscle fibers slightly. Although this new cross-bridge model differs from our previous cross-bridge model in certain respects, two of the major premises of the earlier model remain the same. First, in both models the transition from the 90° to the 45° state is mainly responsible for the recovery of force in the isometric transient. Second, in both models the transition from the refractory to the non-refractory state has a major influence on both the force-velocity curve and the ATPase rate. A completely quantitative version of our earlier cross-bridge model is in press in Biophys. J. The qualitative version of our new

cross-bridge model will be published in Annual Reviews of Physiology in 1980 as part of a review article on the relationship between muscle biochemistry and physiology.

In addition to this work on cross-bridge models, we are analyzing in detail the entropy and energy changes involved in the binding of the two HMM heads to actin. After the first HMM head binds in a second order reaction, the second head binds in a first order reaction. This latter reaction is of interest for several reasons. First, it is a first-order reaction like the binding of the cross-bridge to actin in muscle. Second, the strong binding of the second head to actin implies that the HMM heads themselves are elastic. This elasticity may be related to the elasticity of the cross-bridge in muscle.

Proposed Course of Project: We will begin to develop a quantitative version of our new cross-bridge model. We will also continue our thermodynamic analysis of the binding of the two HMM heads to actin *in vivo* and the binding of the cross-bridge to actin in muscle.

Publication:

Eisenberg, E. and Greene, L.E.: The relation of muscle biochemistry to muscle physiology. Annual Review of Physiol. in press.

Eisenberg, E., Hill, T.L., and Chen, Y.: A cross-bridge of muscle contraction: quantitative analysis. Biophys. J. in press.

Hill, T.L. and Eisenberg, E.: Simplified theory of the Huxley-Simmons T_0, T_1, and T_2 in muscle models with two attached states. In: The Role of Cross-Bridges in Muscle, Tokyo, University of Tokyo Press, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00501-05 LCB

PERIOD COVERED

October 1, 1978 to September 30, 1979

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

Esther Reichstein	Visiting Fellow	LCB NHLBI
Stephen Mockrin	Staff Fellow	LCB NHLBI
Edward D. Korn	Chief	LCB NHLBI

COOPERATING UNITS (if any)

None

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Laboratory of Cell Biology

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National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

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1.5	1.5	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A protein analogous to profilin previously isolated by others from human platlets and calf spleen has been purified from Acanthamoeba castellanii. The protein consists of a single polypeptide chain of molecular weight about 12,000-14,000. It probably forms a 1:1 molar complex with monomeric actin and inhibits the rate of polymerization of actin by inhibiting the first step in the polymerization process - the nucleation reaction in which approximately 3 actin monomers interact to form a nucleus that can be elongated by addition of other actin subunits. Profilin does not seem to inhibit the elongation steps nor the final extent of polymerization. Acanthamoeba profilin has been shown to be present in sufficient quantities in the cell to account for all of the non-polymerized actin, about 50% of the total actin in the cell. Acanthamoeba profilin has a pI of about 6.4 and contains about 28% α-helix and 29% β-conformation. This is the first isolation of profilin from a non-mammalian source. The isolation procedure (40% recovery) should be applicable to the isolation of similar proteins from other sources. Profilin is probably one important element in the processes by which the time and place of actin polymerization in the cell is regulated.

Project Description

Objectives: Actin is one of the major proteins of the cytoskeleton of all eukaryotic cells. It is definitely involved in many motile activities of the cells and in the maintenance of cell shape and organization. Actin itself is a globular protein of 42,000 daltons that polymerizes into double-stranded helical filaments under ionic conditions similar to those in cells. Nonetheless, we have previously shown that non-muscle cells contain about 100 times the concentration of non-polymerized actin that would be expected from the properties of the pure protein. This implies the presence of a protein in non-muscle cells that can interact with actin to prevent, or regulate, its polymerization. Last year, we obtained evidence for such a protein in Acanthamoeba castellanii but had not purified it sufficiently to compare to a similar protein that had been isolated by others from calf spleen. It was our major purpose in this project, to purify the putative protein to homogeneity and to characterize it and its interaction with actin.

Methods employed and major findings: Extracts of Acanthamoeba were fractionated by a sequence of steps including DEAE-ion exchange chromatography, ammonium sulfate precipitation, adsorption on hydroxylapatite and gel filtration of Sephadex G-150. Fractions were monitored for their ability to inhibit the rate of polymerization of G-actin to F-actin. Polymerization was followed by either the increase in viscosity or the increase in absorption at 232 nm that accompanies polymerization. The concentration of non-polymerized G-actin was independently measured by its ability to inhibit the enzymatic activity of pancreatic DNase I with which it forms a tight complex.

A protein, Acanthamoeba profilin, has been purified to homogeneity in approximately 40% yield. This protein is a single polypeptide of about 12,000-14,000 daltons with an amino acid composition that is similar to, but different from, the previously described mammalian profilin. Circular dichroism studies show the molecule to contain about 28% α-heliz and 29% β-conformation. Actin polymerization is a two-step process, (1) the nucleation step in which approximately 3-4 actin monomers interact, and (2) the elongation step in which actin monomers add to the nuclei to form very long filaments. The nucleation step is the slow, rate-determining step in the polymerization process. We have been able to show that profilin inhibits the rate of nucleation but not the rate of elongation by demonstrating that the inhibition of polymerization of actin by profilin can be reversed by the addition of a very small amount of actin nuclei (sonicated fragments of F-actin). Present evidence suggests that, as in the mammalian system, actin and profilin form a 1:1 complex. On this basis there is sufficient profilin in Acanthamoeba to account for all of the non-polymerized actin in the cells. It thus seems likely that profilin is a major component of the regulatory mechanism that controls the state of actin in the amoeba.

Proposed course of research: Several different approaches will be explored as a means of studying the nature of the interaction between actin and profilin. G-actin contains one mole of bound nucleotide. The content of bound nucleotide of the actin-profilin complex will be measured. If it contains nucleotide, the rate of exchange of that nucleotide will be compared to the rate of exchange of the nucleotide bound to G-actin. Actin and profilin will be labelled with reporter molecules that give fluorescence or electron spin resonance signals to monitor the interaction of the two proteins. Suitable chemical modifications will be undertaken in attempts to identify the active sites on both proteins required for their interaction. The reconstituted complex of actin and profilin is not as stable as the naturally occurring complex. Efforts will be made to determine if this is due to inactivation of the profilin during its isolation, to missing components in the reconstituted system or to other factors.

Publications

1. Yang, Y.-Z., Korn, E.D., and Eisenberg, E.: Binding of tropomyosin to copolymers of Acanthamoeba actin and muscle actin. J. Biol. Chem. 254: 2084-2088, 1979.

2. Yang, Y.-Z., Korn, E.D., and Eisenberg, E.: Cooperative binding of tropomyosin to muscle and Acanthamoeba actin. J. Biol. Chem., in press.

3. Reichstein, E. and Korn, E.D.: Acanthamoeba profilin. A protein of low molecular weight from Acanthamoeba castellanii that inhibits actin nucleation. J. Biol. Chem. 254: 6174-6179, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00502-02 LCB

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Acanthamoeba myosins heavy chain kinases

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

Jimmy H. Collins	Staff Fellow	LCB NHLBI
Edward D. Korn	Chief	LCB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

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Section on Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

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0.7	0.7	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

We have previously identified and partially purified a kinase from Acanthamoeba that catalyzes specifically the phosphorylation of the heavy chains of three related enzymes, Acanthamoeba myosins IA, IB and IC. Phosphorylation of their heavy chains had also been shown to be required for actin-activation of the Mg^{2+}-ATPase activities of these three myosins. We have now shown a direct correlation between the extent of phosphorylation of the heavy chains of Acanthamoeba myosin IA and its actin-activated Mg^{2+}-ATPase activity over a range of 0 to 0.8 moles phosphate per mole of heavy chain. We have now purified another kinase that specifically phosphorylates the heavy chain of a different myosin isoenzyme, Acanthamoeba myosin II. However, phosphorylation of the heavy chain, in this case, has no demonstrable effect on the actin-activated Mg^{2+}-ATPase activity of the myosin. ^{32}P is incorporated into the heavy chain of myosin II when cells are grown in the presence of [^{32}P]phosphate and myosin II that is labelled in this way (1 mole ^{32}P/mole heavy chain) does not incorporate additional phosphate when incubated with the kinase. Thus, the phosphorylation of myosin II heavy chain occurs physiologically but its role is not known.

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Project Description:

Objectives: We had shown last year that three of the myosin isoenzymes of Acanthamoeba castellanii, myosins IA, IB and IC, are specifically phosphorylated in their heavy chains by a kinase that was partially purified from cell extracts. This phosphorylation of the heavy chain was necessary and sufficient for actin-activation of the myosin Mg^{2+}-ATPase activity of these three single-headed myosin isoenzymes. Acanthamoeba also contains a more typical double-headed myosin, myosin II, which we have previously shown to be a different gene product than the single-headed myosins. However, its Mg^{2+}-ATPase activity was not activatable by actin. By analogy with the heavy chain kinase for the myosin I isoenzymes, we assumed that there would be a myosin II heavy chain kinase and that phosphorylation of the myosin II heavy chain would allow actin-activation of its Mg^{2+}-ATPase activity. In this way, the cell would regulate its motility mechanisms. The primary purpose of the present project, therefore, was to identify the putative myosin II heavy chain kinase.

Methods employed and findings: Kinase activity was assayed by incubating purified myosin II with the protein fractions to be tested together with [γ-^{32}P]ATP and measuring the incorporation of ^{32}P into the myosin by precipitating the protein onto filter paper and counting in a scintillation counter. Alternatively, the specific localization of the incorporated ^{32}P was determined by separating the myosin subunits by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, slicing the gels and comparing the distribution of radioactivity to the scans of the gels previously stained with Coomassie blue. Standard procedures for purification were employed such as DEAE-cellulose chromatography gel filtration, adsorption on hydroxyapatite and affinity chromatography on ADP-agarose.

We succeeded in partially purifying a kinase that could incorporate 1 mole of phosphate into each of the heavy chains of myosin II. The enzyme had no activity towards any of the subunits of myosins IA or IB nor did it phosphorylate any of the subunits of rabbit skeletal muscle myosin. It did phosphorylate the isolated 20,000-dalton light chain of chicken gizzard smooth muscle myosin. This myosin II heavy chain kinase phosphorylated band 2 of erythrocyte spectrin but not band 1 (the very different enzyme, myosin I heavy chain kinase, phosphorylated both bands 1 and 2 of spectrin and the heavy chains of myosins I but not the heavy chain of myosin II).

However, in contrast to our findings with phosphorylation of Acanthamoeba myosins I, complete phosphorylation (1 mol/mol) of the heavy chains of Acanthamoeba myosin II did not enhance the ability of actin to activate its Mg^{2+}-ATPase activity. To test whether this heavy chain kinase activity might be an artifact and not represent a true cellular activity, we grew Acanthamoeba in the presence of [^{32}P]phosphate and rapidly isolated the myosin II and separated its heavy chain. The heavy chain had incorporated 1 mol of ^{32}P per mol of heavy chain. This myosin was no longer a substrate for the partially purified myosin II heavy chain indicating that the isolated enzyme was the one that functions in the cell. However, even the myosin II that was phosphorylated in its heavy chain in situ was not activated by actin. Therefore,

the mechanism for regulating Acanthamoeba myosin II's actin-activated ATPase activity is still unknown.

Proposed course of research: The actin-activated ATPase activities of vertebrate smooth muscle and non-muscle myosins is known to be regulated through phosphorylation of one of the light chains of the myosins. This differs, of course, from the regulation of Acanthamoeba myosin I by phosphorylation of its heavy chain. We hope to explore the possibility that Acanthamoeba myosin II may, by analogy to the vertebrate myosins, be regulated through phosphorylation of one of its light chains and, therefore, differ significantly in this respect, as in others, from the myosin I isoenzymes in the same cell. We would also hope to determine what is responsible for the enhanced actin-activation of phosphorylated Acanthamoeba myosin I and the role of heavy chain phosphorylation in Acanthamoeba myosin II.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00503-07 LCB

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Structure, Assembly and Function of Microtubules

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

PI:	Martin Flavin	Head, Sec. on Organelle Biochemistry	LCB NHLBI
OTHER:	Takaaki Kobayashi	Visiting Fellow	LCB NHLBI
	Nirbhay Kumar	Visiting Fellow	LCB NHLBI
	Todd Martensen	Staff Fellow	LCB NHLBI
	Jayasree Nath	Visiting Associate	LCB NHLBI
	Terry Jones	Biologist	LCB NHLBI

COOPERATING UNITS (if any)

Dr. William Adelman, Laboratory of Biophysics, NINCDS

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National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

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4	4	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

We have been studying a post-translational modification of tubulin (the protein building block of microtubules) which we think may regulate microtubule assembly or function. This involves the reversible enzymatic addition of a tyrosine residue to the C-terminus of the α chain. An improved procedure has been devised for purifying the enzyme which adds tyrosine. A separable detyrosylating enzyme has been purified 500-fold from brain and has been shown to possess some distinctive properties unlike those of other carboxypeptidases. Attempts have been made to reconcile our previous, apparently contradictory, findings that membrane bound tubulin from brain is isolated without any C-terminal tyrosine, yet tubulin from a particulate fraction from cultured neuronal cells becomes tyrosylated in vivo.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: We are investigating an enzymatic reaction by which a tyrosine residue can be reversibly added to the C-terminus of the α chain of tubulin. Our objective is to discover what this post-translational modification is for. Because it is unprecedented, we suspect that it will prove to have an important function in regulating microtubule assembly or function, either directly or by influencing the partition of tubulin among subcellular compartments.

Methods Employed: Biochemical procedures as indicated under Major Findings.

Major Findings

a. Tubulin-tyrosine ligase: Purified preparations of this brain enzyme, which catalyzes the reversible addition of tyrosine to the α chain of tubulin, are essential for the preparation of completely tyrosylated tubulin, and for assays of the extent to which tubulin samples are tyrosylated. Considerable time has been spent on revising the purification procedure, since the method described 2 years ago could not be reproduced, in part perhaps because the responsible coworker left the laboratory without a detailed description. Also, because the commercial source had discontinued agarose gel with propylamine substituents (used for coupling to tyrosine for affinity chromatography) gels with other spacer groups were tried, but without success. We have now again obtained the original agarose derivative, and by modifying the DEAE-cellulose step and introducing a new phospho-cellulose step we have developed a reproducible procedure for 200-fold purification.

b. Detyrosylating enzymes: The ligase catalyzes detyrosylation on addition of ADP + Pi but (as described last year) brain extracts also contain another detyrosylating enzyme (CPT) which may be a carboxypeptidase specific for tubulin. CPT has been purified 500-fold by Todd Martensen by salt fractionation, and DEAE- and carboxymethyl-cellulose chromatography. This preparation detyrosylates tubulin without further digesting the α chain, as does carboxypeptidase A, and has distinctive properties. When assayed with denatured (reduced and alkylated) α chain as substrate, it is inhibited 90% by 1.5 mM MgGTP, 40% by 1 mM colchicine, and 100% by 1 mM trifluoperazine. It has a molecular weight of 74,000 ± 2000, and has hydrophobic properties, like the low molecular weight forms of the ligase. The purified enzyme does not show those properties reported last year for crude preparations which suggested that it might act preferentially on tubulin in assembled microtubules. Assays are difficult in extracts because of the low specific activity of CPT.

c. Effects of tyrosine and tubulin on the electrical properties of the perfused squid axon: The rationale for this approach was given in last year's "proposed course". Other suggestive observations are that tubulin is present in the membrane of cilia whose motility is electrically coordinated, but absent from flagellar membranes; and membrane-bound tubulin from brain is known to differ from the cytoplasmic in only 1 respect, the absence of C-terminal tyrosine. With Dr. William Adelman at Woods Hole apparatus was designed providing reduced dead space for small volumes of perfusate, and

cooling of cannula and perfusate to prevent microtubule assembly with obstruction of flow. Perfused axons were subjected to rapid stimulation until the action potential began to fail. While continuing the stimulation addition of ATP + Mg + tyrosine to the perfusate had no effect, but further addition of tubulin restored the action potential. Due to the seasonally poor quality of squid, the experiment was done successfully only once.

d. Differences in tyrosylation between cytoplasmic and membrane-bound tubulin of brain. Prior results of Jayasree Nath showed that tubulin solubilized from a brain membrane fraction had no C-terminal tyrosine, although it was a good ligase substrate and otherwise was indistinguishable from cytoplasmic tubulin. On the other hand, in studying the turnover of α chain tyrosine in living cultured neuronal cells, i.e. in the absence of protein synthesis, we had found that tyrosine was fixed in the particulate as well as soluble fractions. The particulate radioactivity was in α chains and unknown component(s) of low molecular weight (but not products of α chain proteolysis). A membrane fraction, similar to that from brain, was isolated from the cultured cells, but the tubulin content was too low to allow us to purify it and determine whether it also appeared to have no pre-existing tyrosine, like brain membrane tubulin.

We therefore turned to studies of α chain turnover in vivo in brain. The first experiments were done by sequential intracerebral injection of protein synthesis inhibitors, followed by labeled tyrosine, in newly hatched checks. Prior to doing these experiments we showed that cytoplasmic and membrane tubulin were similar in chicks and rats and chick membrane tubulin also appeared to have no C-terminal tyrosine. Controls were also done to show that no cytoplasmic tubulin was trapped in the membrane preparation, and no detyrosylation occurred during extraction and purification of tubulin from this fraction. Pure ^{14}C-tyrosyltubulin added to the membrane fraction was not subsequently detyrosylated. A third of it spun down when this fraction was centrifuged; curiously as much or more spun down with the much reduced pellet obtained after detergent "extraction" of the endogenous tubulin.

From the intracerebrally injected chicks, membrane and cytoplasmic tubulin were extracted and purified exactly as before. Gel electrophoresis and fluorography showed that α chain was the only labeled component (except for a little low molecular weight material from the membrane fraction), and that membrane and cytoplasmic tubulin had indeed acquired comparable amounts of labeled C-terminal tyrosine.

To obtain more extensive tyrosine turnover, experiments were done with minced brain under the same conditions previously used for cultured neuronal cells, i.e. a membrane fraction was not isolated, and the whole soluble and particulate fractions were applied directly to polyacrylamide gels. Since in these experiments 30% of the added radioactivity was intracellular after the incubation (compared with 1% after intracerebral injection), it was possible to make a rough estimate of the extent of α chain C-terminal tyrosine turnover: the calculated values were about 0.05 nmoles tyrosine per nmole tubulin for both soluble and particulate fractions.

Our current thoughts about this apparent paradox are roughly as follows. 1) Explanations at least partly already ruled out: the membrane has both species but the tyrosylated tubulin is not extracted by Nonidet; tubulin already in the membrane is detyrosylated during isolation; when membrane tubulin is tyrosylated it becomes susceptible to protease digestion. 2) The carboxypeptidase A and ligase assay method we use to measure pre-existing tyrosine is not valid - in this assay, tubulin might appear to have no pre-existing tyrosine if there were ligase-catalyzed exchange between free and fixed tyrosine; such an exchange might occur if sufficient ATPase were present in the tubulin purified from membrane extracts. 3) Exogenous labeled tyrosine reaches the membrane without being diluted by an intracellular pool or catabolized to other products, i.e. in membrane tubulin a lot of label represents a little tyrosine.

e. Miscellaneous: During current studies by Jayasree Nath of tyrosylation states of tubulin in virally transformed and untransformed 3T3 cells, we noted that both cell types resemble HeLa in failing to turn over any α chain tyrosine in vivo.

Proposed Course of Project

A continuing objective is to prepare labeled tubulin which is either completely detyrosylated or maximally tyrosylated, and compare the binding of the 2 species of dimeric or assembled tubulin to relevant macromolecules, cell organelles, adenoviruses etc. Studies of binding to calmodulin have already been initiated by Todd Martensen.

We will try to extend the ligase purification at least to a point where the complete reaction products can be identified, and to determine the subcellular localization of it and the detyrosylating enzyme, CPT. We would like to ascertain the specificity of the latter with regard to protein substrate, and perhaps explore further the question of whether the crude enzyme differs from the purified in acting preferentially on assembled microtubules. In our hands colchicine has enhanced α chain turnover in vivo, but another laboratory has reported inhibition and concluded that assembly is required at some stage of the turnover.

Other continuing objectives are to identify the structural features (i.e. possibly β chain phosphorylation) which prevent some portion of tubulin from serving as a ligase substrate, and to correlate changes in tubulin tyrosylation state in different cells, and in different subcellular compartments, and in tubulin in different states of assembly.

Publications

Nath, J., and Flavin, M.: A structural difference between cytoplasmic and membrane-bound tubulin of brain. FEBS Letters 95: 335-338, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00505-13 LCB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Cytology of Acanthamoeba

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

PI:	Blair Bowers	LCB NHLBI
OTHER:	Enrico Cabib	LBM NIAMD
	Jesus Molano	LBM NIAMD
	Angel Duran	LBM NIAMD
	John E. Hyde	HV BR NHLBI

COOPERATING UNITS (if any)

Laboratory of Biochemistry and Metabolism, NIAMD; Biometrics Research Branch, DHVD, NHLBI

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Laboratory of Cell Biology

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National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

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3	1	2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

We are studying membrane recirculation accompanying endocytosis in Acanthamoeba castellanii using quantitative electron microscopy. We plan to continue this line of investigation using labelled antibodies to a membrane glycolipid as a specific probe for following membrane recirculation.

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Project Description

Objectives: Our major project is a study of how surface membrane taken in during endocytosis is handled by the cell and in what form it is returned to the surface. We are using as a model system a small amoeba, Acanthamoeba castellanii, a cell that has an unusually high rate of membrane recirculation associated with pinocytosis. The problem is most accessible through morphologocial techniques that examine the relevant membranes in situ, so that a variety of electron microscopic procedures are being utilized.

Methods Employed: Transmission electron microscopy is being used for study of fixed and embedded cells and for examination of purified proteins by negative staining. Scanning electron microscopy is being used for examination of surface topography.

Quantitative analysis of electron micrographs is being performed using the methods of stereology.

Major Findings

1. Acanthamoeba provides a valuable system for study because endocytosis and the accompanying membrane recirculation is highly exaggerated relative to mammalian cells. Three morphologically distinguishable "compartments" comprise the major part of recirculating membrane in Acanthamoeba; they are the plasma membrane, vacuolar membrane and small vesicle membrane. Experiments were designed to obtain quantitative estimates of the amounts of membrane in these three interrelated and exchangeable compartments, and, further, to follow changes in the amount of membrane in each compartment when the rate of recirculation was decreased by causing the cells to switch from pinocytosis to phagocytosis. Cell samples were taken for electron microscopy 0, 15, 30, and 45 min. after presenting the cells with saturating loads of particles. The surface areas and volumes of the compartments were measured by morphometric analysis of electron micrographs taken at each of the four time periods. Last year we reported the results of the analysis of two separate experiments. This year we completed the analysis of a third experiment and performed the statistical analysis of all the results. The major conclusions from the first two experiments were confirmed by the third, and the data addition from another experiment allowed us to test the statistical significance of the observed changes.

The results of the complete study show that the plasma membrane surface area increases transiently during the first 30 min. (a period when the rate of surface internalization by endocytosis is decreased). This is interpreted to mean that the influx and efflux of surface membrane are not tightly coupled or completely reciprocal processes.

The surface area of "empty" vacuoles, found in the cytoplasm of pinocytosing cells, diminishes at a rate that is proportional to the rate of formation of phagosomes, suggesting that the two events are related in some way; however, there does not appear to be a one for one exchange at the cell sur-

face of an empty vacuole for a phagosome. There is, on the other hand, evidence for fragmentation of the empty vacuoles, since the average surface to volume ratio increases during the experiment (3.2, 3.7, 5.1, and 3.9 for 0, 15, 30, and 45 min. respectively), while that of the phagosomes remains constant at 1.8. The vacuolar membrane surface area remains approximately constant despite a demonstrable exchange of most of the membrane in the compartment during the 45 min. period of the experiment. This finding implies that the membrane of the vacuolar compartment turns over, like the plasma membrane, but is more closely regulated by the cell that is surface membrane.

The area of membrane in the small vesicle compartment declines sharply during phagocytosis. The vesicle compartment accounts for 43% of the total membrane of the three compartments at 0 time. After 45 min. of phagocytosis only 27% of the membrane is found in small vesicles. This compartment appears to be derived both from small pinocytic vesicles moving in and fragmentation of the vacuolar membrane destined to move to the cell surface. These observations, and other morphological data reported previously, suggest that membrane recirculating to the surface moves back through the small vesicle pool.

2. Two new projects have been initiated in collaboration with H. Gadasi, S. Brenner, and E.D. Korn. The purpose of these projects is to examine by transmission electron microscopy some of the interactions of molecules that are being studied biochemically by these investigators. The interaction are those of Acanthamoeba myosins with actin and of erythrocyte spectrin with actin. We are also attempting to demonstrate the structure of the small Acanthamoeba myosin 1A by electron microscopy. Considerable time has been spent during the current year investigating the conditions necessary for successful examination of these molecules by negative staining and shadowing, but no definitive results have been obtained.

3. As part of a continuing collaboration with Enrico Cabib and his laboratory, in studies on chitin localization and the control of chitin synthesis on the year, Saccharomyses cerevisiae, we have demonstrated the distribution of chitin synthase on the yeast cell plasma membrane by electron microscopic autoradiography. Purified, intact plasma membranes isolated from protoplasts were allowed to synthesize chitin in the presence of UDP-N-[^{3}H]acetylglucosamine. The chitin itself is invisible in the electron microscope preparations because it does not take up electron-dense stains and has the same electron capacity as the embedding plastic. Nevertheless, the location of the chitin synthesized from the tritiated substrate can be determined by the distribution of developed silver grains in autoradiograms of membrane preparations washed free of unincorporated substrates. The results of these experiments showed that silver grans were randomly distributed along the entire length of the plasma membrane profiles. In vivo chitin synthase activity is manifest only in a specific location and at a specific time when the primary septum is laid down. The distribution shown in autoradiograms suggests that chitin synthase (known to be a zymogen) occurs over the entire membrane, but is activated at specific sites on the membrane when the septum is to be laid down.

In a separate study with E. Cabib and J. Molano we attempted to identify more precisely the location of chitin in the yeast cell wall by use of specific electron-opaque labels for the chitin. The experiments had the purpose of confirming and refining localizations previously made by biochemical means, and also of developing labels that can be used on thin sections in order to study the timing of chitin synthesis in relation to the budding process. Labels for chitin were prepared by coating (stabilizing) colloidal gold of suitable dimensions ($\sim$ 15 nm) with chitin-specific proteins. Three ligands were used: wheat germ agglutinin, specific for N-acetyl glucosamine, and 2 chitinases, extracted from different sources, which are highly specific but less stable ligands than the wheat germ agglutinin.

The interactions of the three colloidal gold labels with several different kinds of preparations were examined by transmission electron microscopy. The preparations were: pure chitin, synthesized in vitro by a purified chitin synthetase; isolated bud scars (largely chitin); cell walls from which the covering layer of mannan had been extracted; extracted cell walls further treated with chitinase or β 1-6 glucanase to selectively remove wall polymers. Control studies indicated that neither colloidal gold itself nor gold coated by a non-specific protein, such as bovine serum albumin, bound to these preparations. In another type of control, oligosaccharides of chitin markedly reduced the binding of the specifically labeled gold.

The chitin labels bound strongly to pure chitin and to bud scars. The bud scars showed uniform labeling, indicating that chitin was distributed over the entire septum. The cell walls (including bud scars) showed a lighter labeling that was generally distributed. The wall label, but not the bud scar label could be removed by short digestion with chitinase, or long digestion with glucanase. Biochemical experiments performed by E. Cabib proved that chitin sugars were released during these digestions, and showed that the chitin found outside the bud scars accounts for less than 10% of the total chitin. These and other experiments suggest that short chains of chitin are attached to the glucan and small amounts of chitin are distributed over the entire wall. The significance, if any, of these small amounts of chitin present in the wall is not yet known.

Proposed Course of Project: We intend to make antibodies to a membrane glycolipid found in Acanthamoeba in order to use labelled antibodies as probes for following membrane recirculation.

Publications

Duran, A., Cabib, E., Bowers, B.: Chitin synthetase distribution on the yeast plamsa membrane. Science 203: 363-365, 1979.

Cabib, E., Duran, A. and Bowers, B. Localized activation of chitin synthetase in the initiation of yeast septum formation. In Trinci, A.J.P. (Ed.): Symposium on Wall and Hyphal Growth. British Mycological Society. Cambridge, U.K., Cambridge University Press, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00506-04 LCB

PERIOD COVERED
October 1, 1978 to September 30, 1979

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:	Hiroshi Maruta	Visiting Associate	LCB NHLBI
	Hana Gadasi	Visiting Fellow	LCB NHLBI
	Sueo Matsamura	Visiting Fellow	LCB NHLBI
	Jimmy Collins	Staff Fellow	LCB NHLBI
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COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Section on Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.7	3.7	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)

Previously, we had discovered that Acanthamoeba castellanii contains three myosin isoenzymes, a double-headed myosin II and single-headed myosins IA and IB, that are products of different genes. The earlier evidence was based largely on the dissimilarity of one dimensional peptide maps of the products of enzymatic and chemical cleavage of the myosins. These data have now been extended by immunochemical studies which show that there are no common antigenic sites between myosin II and either myosin IA or IB. Myosin IA and IB do show some cross-reactivity but they can be differentiated immunologically. In addition, we have found no evidence for higher molecular weight peptides that cross-react with the antibodies directed against the heavy chains of myosins II, IA and IB. Therefore, it seems increasingly likely that, despite their low molecular weights, these are the functional forms of the enzymes in the cell. The low molecular weight of myosin IA has been definitively confirmed as 150,000 by a combination of sedimentation-equilibrium and sucrose density gradient centrifugation. For the first time, we have obtained preparations of myosin II whose Mg-ATPase activity can be activated by F-actin. Finally, evidence has been obtained for an actin-activatable myosin that may be a single-headed form of myosin II.

Objectives: We had previously discovered that extracts of Acanthamoeba castellanii contain four separable myosin ATPases: myosin II, a double-headed enzyme of molecular weight about 400,000 consisting of a pair of heavy chains of 170,000 daltons and two pairs of light chains of 17,500 and 17,000 daltons; myosin IA, a single-headed enzyme of molecular weight about 180,000 consisting of one heavy chain of 130,000 daltons and two light chains of 17,000 and 14,000 daltons; myosin IB, a single-headed enzyme of molecular weight about 180,000 consisting of one heavy chain of 125,000 daltons and two light chains of 27,000 and 14,000 daltons; and myosin IC, a single-headed enzyme of molecular weight about 180,000 consisting of one heavy chain of 130,000 daltons and three light chains of 20,000, 17,000 and 14,000 daltons. We had shown that myosin IC was the same as myosin IA except for the presence of the additional 20,000-dalton light chain and that myosin II and myosin IA(IC) were products of different genes. Myosins IA, IB and IC were similar enzymatically and, most interestingly, their Mg-ATPase activities were activated by actin after phosphorylation of their heavy chains by a specific myosin I heavy chain kinase. An actin-activatable form of myosin II had not been obtained although myosin II had been shown to bind to F-actin. Among our major objectives for this year were the following: (1) to confirm the unusually low molecular weights of the single-headed myosins I; (2) to determine if myosin IB was related or unrelated to myosin IA; (3) to confirm by an independent means that the several Acanthamoeba myosins were, in fact, products of different genes, i.e. true isoenzymes, and not derived from a common precursor; (4) to prove that the myosin isoenzymes were all present in a single cell rather than being derived from different cells in a mixed population; (5) to obtain a form of myosin II whose enzymatic activity could be activated by actin; (6) to characterize a fifth, minor component of the amoeba extract that seemed to have myosin-like enzymatic properties.

Methods Employed and Major Findings: (1) Molecular weights of myosin I: Highly purified myosin IA, the myosin I isoenzyme obtainable most readily and in highest yield, has been analyzed by sedimentation-equilibrium viscosity and sucrose gradient centrifugation. This combination of techniques allows a calculation of the shape of the molecule (axial ratio) and its molecular weight. The results indicate a molecule of axial ratio about 12:1 and a molecular weight of 150,000. This molecular weight is in good agreement with the sizing by gel filtation (approximately 180,000) and the sum of its subunits as determined by sodium dodecyl sulfate-polyacrylamide electrophoresis on the assumption of one polypeptide chain each of molecular weights 130,000 and 17,000. There is also a small amount of a third polypeptide of molecular weight 14,000 which may, or may not, be an integral component of the enzyme. The subunits would add up to 147,000 or 161,000 depending on whether the 14,000-dalton component is included. These data establish that myosin IA, and by inference myosin IB and IC, are as we believed, single-headed enzymes.

Relation of myosin IB to myosin IA(IC): By use of the same techniques that established that myosin IA(IC) is a different gene product from myosin II, we have found that myosin IB is the product of yet a third gene. Highly purified myosin IB was partially cleaved at methionine residues by cyanogen bromide and

at cysteine residues by the cyanylation reaction and the peptides produced were compared to peptides produced in similar reactions from myosin IA and myosin II. Single dimension sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed there were no peptides in common. Similarly, the isolated heavy chains of myosin IB (125,000 daltons), myosin IA (130,000 daltons) and myosin II (170,000 daltons) were degraded by Staphylococcus aureus protease and chymotrypsin and peptide maps of the products compared. Again no common peptides were observed. Therefore, myosin IB heavy chain is a product of a third myosin heavy chain gene and Acanthamoeba contains three myosin isoenzymes: myosin II, myosin IA(IC) and myosin IB.

(3) Confirmation of myosin isoenzymes: Antibodies were raised in rabbits to purified myosin II, IA and IB. The antisera were purified to the IgG fractions. These were then assayed for antibody reaction and specificity in the radio-immunoassay using ^{125}I-labeled myosins. In this reaction small amount of the ^{125}I-myosin are incubated with the suspected rabbit IgG antibody fraction and then an excess of burro anti-rabbit IgG is added to precipitate quantitatively any complex that had formed between the ^{125}I-myosin and the rabbit IgG. The results clearly showed that antibodies raised against myosin II reacted with ^{125}I-myosin II but not with ^{125}I-myosin IA or IB. Neither anti-myosin IA nor anti-myosin IB reacted with ^{125}I-myosin II. Similarly, anti-myosin II inactivated the actin-activated myosin II ATPase but had no effect on the actin-activated myosin IA ATPase activity while anti-myosin IA and anti-myosin IB inactivated actin-activated myosin IA ATPase but not actin-activated myosin II ATPase. These data confirm that myosin II is a product of a different gene than are myosins IA and IB since they have no antigenic sites in common. Myosin IA and myosin IB clearly share at least some antigenic sites. However, in competitive radioimmunoassays in which the ability of non-radioactive myosin to compete in the antigen-antibody reaction using ^{125}I-myosin is tested, clear differences in specificity could be shown between the antibodies raised against myosin IA and IB. Thus, myosin IA and IB seems to be antigenically distinct. That they should have similarities is not unexpected since they are enzymatically much more similar than either is to myosin II, and they are both substrates for the same myosin I heavy chain kinase. Direct precipitation of polypeptides from extracts of whole amoebae with the several IgG fractions followed by electrophoretic analysis of the precipitated proteins also demonstrated this specificity. Anti-myosin II IgG precipitated only a polypeptide corresponding to the heavy chain of myosin II (in that molecular weight range) while anti-myosin IA precipitated only the heavy chain of myosin IA and anti-myosin IB precipitated only the heavy chain of myosin IB. Finally, the proteins of whole amoebae were separated electrophoretically on polyacrylamide gels which were then reacted sequentially with one of the three antisera followed by ^{125}I-protein A (a molecule that reacts with all IgG molecules). Among polypeptides of high molecular weight, anti-myosin II reacted only with a polypeptide of 170,000 daltons (the myosin II heavy chain) while anti-myosin IA and IB both reacted with the heavy chains of myosins IA and IB but with no other proteins. Thus, these immunochemical data support the idea that myosin IA, IB and II are products of different genes and give further support to the belief that, despite their low molecular weights relative to other myosins, these isolated enzymes are the functional forms that exist in the cell.

300

(4) Are the three myosin isoenzymes present in the same cell? The amoebae were cloned by selecting single cells under the microscope and culturing them separately. In this way, three different populations of amoebae were raised from single cells and their myosin contents determined by purifying the several enzymes in the usual way. In all three cases, myosin IA, IB and II were present in the same ratio as in the parent population. This establishes that the three myosin isoenzymes are all present in the same cell. As a side benefit, one of these clones was found to grow more rapidly and to greater cell densities than the parent population and is now our stock culture.

(5) Actin-activatable myosin II: Until recently all attempts to obtain myosin II in a form such that its Mg-ATPase would be activated by F-actin had failed. This was very important because in any model for motility based on the proteins actin and myosin it is almost a necessity that the ATPase of the myosin be enhanced when it is coupled to F-actin. For smooth muscle and vertebrate non-muscle myosins, actin-activation depends on the phosphorylation of one of the two light chains of the myosin by a specific kinase. For Acanthamoeba myosins IA, IB and IC, we have previously shown that actin-activation depends on the phosphorylation of the heavy chains by a specific kinase. Phosphorylation of the heavy chain of myosin II occurs but does not result in actin-activation. Recently, we have isolated myosin II by a new procedure based on one developed by Spudich at Stanford University for myosin from Dictyostelium discoideum. Three of five preparations have been activated by actin to about 50-fold with specific activities of nearly 1 μmol/min/mg of protein, values about as high as have been obtained for the actin-activated Mg-ATPase activity of any non-muscle myosin other than the Acanthamoeba myosins I. We do not yet know why myosin II isolated in this way behaves differently. It has the same native molecular weight and subunits of the same size by acrylamide gel electrophoretic analysis.

(6) Characterization of a minor myosin-like ATPase fraction: We have obtained evidence that amoeba extracts contain a protein that resembles myosin enzymatically and that has a native molecular weight between that of myosin I and myosin II. Its Mg-ATPase activity is activated by F-actin. The protein has not yet been adequately purified but preliminary evidence is compatible with the assumption that it may be a single-headed form of myosin II. Such a species has been detected by Dr. T.D. Pollard (Johns Hopkins University) in sedimentation equilibrium studies of highly purified preparations of myosin II. It is possible that the dissociation that occurs in the centrifuge may also occur in the cell or at least in the cell extracts.

Proposed course of project: Among the lines we would like to explore in the next year are the following. We would like to develop a reproducible procedure for isolating myosin II in a form such that its Mg-ATPase activity is activated by F-actin. When that has been accomplished it should be possible to determine how this myosin II differs from non-activatable preparations. One likely possibility is that it will have a phosphorylated light chain. We would hope to have established the nature of the partially purified myosin-ATPase that may be a form of myosin II. We would hope to use more highly purified antibodies to establish unequivocally that the isolated forms of

myosins IA, IB and II are the native forms in the cell, to determine if these or similar myosins occur in other organisms, and to localize through fluorescence and electron microscopy the occurence of each of the three myosins in situ. We would also hope, in collaboration with Dr. Blair Bowers of this Laboratory, to determine the shape of myosins I and II in the electron microscope, the properties of the filaments they may form and of their complexes with actin.

Publications:

1. Maruta, H., Gadasi, H., Collins, J.H., and Korn, E.D.: The isolated heavy chain of an Acanthamoeba myosin contains full enzymatic activity. J. Biol. Chem. 253: 6297-6300, 1978.

2. Korn, E.D.: Regulation of the form and function of actin and myosin of non-muscle cells. Acat. Protozoologica 18: 75-90, 1979.

3. Maruta, H., Gadasi, H., Collins, J.H., and Korn, E.D.: Multiple forms of Acanthamoeba myosin I. J. Biol. Chem. 254: 3624-3630, 1979.

4. Gadasi, H., Maruta, H., Collins, J.H., and Korn, E.D.: Peptide maps of the myosin isoenzymes of Acanthamoeba castellanii. J. Biol. Chem. 254: 3631-3636, 1979.

5. Gadasi, H., and Korn, E.D.: Immunochemical analysis of Acanthamoeba myosins IA, IB and II. J. Biol. Chem. in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00507-03 LCB

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Interactions between Spectrin and Actin

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

Stephen Brenner	Staff Investigator	PSL DCRT
Edward D. Korn	Chief	LCB NHLBI

COOPERATING UNITS (if any)

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LAB/BRANCH

Laboratory of Cell Biology

SECTION

Section on Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, 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)

The low ionic strength extract of sheep erythrocyte ghosts has been fractionated by gel chromatography into: a complex of spectrin and actin, spectrin tetramer, spectrin dimer, actin, and hemoglobin. Spectrin heterodimer binds to F-actin but does not crosslink it. Spectrin tetramer can crosslink filaments of F-actin as shown by the large increase viscosity when the two proteins are mixed. The complex seems to induce the polymerization of G-actin to F-actin under condition where the actin would not have polymerized alone. Contrary to previous reports of others, the interactions of spectrin dimer and tetramer with actin are independent of the state of phosphorylation of the spectrin. Identical results were obtained with native spectrin (3-4 phosphates/dimer), spectrin to which 1 mole of phosphate was added by incubation with an erythrocyte protein kinase and ATP, and spectrin from which 85% of the phosphate groups were removed by incubation with E. coli alkaline phosphatase.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The shape and viscoelastic properties of the red blood cell are maintained by a cytoskeletal structure immediately under and associated with the cell membrane. Two major constituents of this structure are the proteins spectrin and actin and their interaction is thought to be an important and possibly regulatable feature of the cytoskeleton. Spectrin is a heterodimer of two large polypeptides (220,000 and 240,000 daltons), the smaller of which contains 3-4 phosphate groups. Circumstantial evidence has accumulated in the literature that suggests that the state of phosphorylation of the spectrin may regulate its interaction with actin. Actin, an ubiquitous protein in eukaryotic cells, is a 42,000-dalton globular protein which, in most cells, is believed to function in the polymeric form of microfilaments of F-actin. The state of the actin, and of spectrin, in erythrocytes is not known but many investigators have speculated that the cytoskeleton may be a network of spectrin tetramer and monomeric G-actin. There is now good evidence that this spectrin/actin network is linked to the red cell membrane through another protein of molecular weight about 200,000 which binds to spectrin and to an integral membrane protein (so-called Band 3). We have undertaken to determine more precisely the nature of the interaction between spectrin and actin with the hope of identifying the polymeric state of both proteins in the complex, the nature of the binding sites and the effect of phosphorylation on their interaction.

Methods Employed and Major Findings: Sheep erythrocytes were washed free of plasma, lysed in a large volume of hypotonic solution and washed free of hemoglobin. These washed erythrocyte ghosts were then extracted for 20 minutes at 37° with a buffer of low ionic strength. The insoluble membrane was removed by centrifugation and the extract fractionated by chromatography on Sepharose 4B. Five fractions were obtained: (1) a complex mostly consisting of spectrin and actin; (2) spectrin tetramer; (3) spectrin dimer; (4) a fraction consisting largely of actin; (5) a fraction consisting of traces of hemoglobin. The interaction of each of these fractions with monomeric G-actin and polymeric F-actin (obtained from rabbit skeletal muscle) was then tested.

Although it had been claimed previously that spectrin was capable of inducing the polymerization of G-actin to F-actin in a non-polymerizing buffer, we found that only the spectrin/actin complex had this property. Neither spectrin tetramer nor spectrin dimer had any measurable interaction with monomeric G-actin as measured either by increase in viscosity that could occur if polymerization were induced or by a reduction of the critical concentration of G-actin necessary for polymerization to occur in a polymerizing buffer. On the other hand, spectrin dimer, which is a heterodimer of polypeptides of molecular weight 220,000 and 240,000, bound to F-actin as measured by its co-sedimentation with F-actin under conditions in which the spectrin dimer would not sediment alone. These studies were facilitated by the use of ^{32}P-spectrin (see below). Spectrin tetramer not only bound to F-actin but could crosslink filaments of F-actin as shown by the large increase in vis-

cosity that occurred when spectrin and F-actin were mixed. The simplest interpretation of these data is that spectrin dimer contains one binding site for actin subunits in F-actin and that spectrin tetramer contains two such binding sites. Actin subunits in F-actin, but not monomeric G-actin, would have spectrin-binding sites.

Previous workers have suggested that the 3-4 phosphate groupes located specifically on the 220,000-dalton peptide of spectrin were important for its interaction with actin. To test this, we labelled spectrin with ^{32}P by incubating erythrocytes overnight with [^{32}P]-phosphate. About 85% of the radioactive phosphate could then be removed from purified spectrin by incubation with E. coli alkaline phosphatase. This dephosphorylated spectrin was quantitatively indistinguishable from native spectrin in its ability to bind to (spectrin dimer) and crosslink (spectrin tetramer) F-actin. Similarly, incorporation of 1 mole of additional phosphate into native spectrin, or several moles of phosphate into dephosphorylated spectrin, by incubation with ATP and an erythrocyte protein kinase had no effect on the ability of the spectrin to interact with actin.

We conclude that the erythrocyte cytoskeleton probably consists of oligomeric actin (not monomeric actin) crosslinked by spectrin tetramer and that this interaction is not regulated by the phosphorylation state of the spectrin.

Proposed course: One of the more intriguing aspects of the recent observations in this and other laboratories is the ability of the complex of spectrin/actin to induce the polymerization of monomeric G-actin. This may be a very useful model for the general problem of the regulation of the polymerization and membrane attachment of actin in non-muscle eukaryotic cells. We intend to study this phenomenon in some detail.

Publications:

1. Korn, E.D.: Preface. In Korn, E.D. (Ed.): Methods in Membrane Biology. New York, Plenum Press, Vol. 10, 1979, pp. ix-x.

2. Korn, E.D. (Ed.): Methods in Membrane Biology, New York, Plenum Press, Vol. 10, 1979, 227 pages.

3. Brenner, S.L., and Korn, E.D.: Spectrin-actin interaction. Phosphorylated and dephosphorylated spectrin tetramer crosslink F-actin. J. Biol. Chem. in press.

ANNUAL REPORT OF THE
LABORATORY OF CELLULAR METABOLISM
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

October 1, 1978 to September 30,1979

As in the recent past, research in the Section on Cellular Regulatory Mechanisms is very 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 the Section on Cellular Pharmacology, studies of histamine metabolism and action along with investigations of the inflammatory process and anti-inflammatory drugs are continuing.

1. Cyclic Nucleotide Phosphodiesterases

We have in the past described a number of mechanisms for the regulation of cyclic nucleotide phosphodiesterase activity in mammalian cells. Recently much of our attention has ben focussed on the soluble phosphodiesterase that is activated by calcium and calmodulin, a calcium-binding protein that is now known to function in several enzyme systems. Extensive purification of cyclic nucleotide phosphodiesterase has been achieved through the development of a new procedure, "sequential adsorption-electrophoresis," which combines adsorption of the enzyme to calmodulin-Sepharose with direct electrophoretic elution and separation from other calmodulin-binding proteins. By applying this technique after two rapid fractionation steps, we are now able to purify the enzyme from brain supernatant 2000- to 3000-fold in three days with an overall yield of 30-40%. (Our previous observations indicating multiple forms of phosphodiesterase probably reflect limited proteolysis and/or fractionation procedures which generate different forms.) The highly purified enzyme exhibits one major band on SDS-gels (MW $\sim$ 58,000) and a lower molecular weight component ($\sim$ 20,000). It is activated $\sim$ sixfold by calmodulin in the presence of calcium which alone has no significant effect. Activation by calmodulin depends upon substrate concentration being more pronounced at low concentrations ($\sim$ 1 µM). The activity toward cGMP is 5-10 times greater than that for cAMP at micromolar concentrations. (The two activities co-elute as a symmetrical peak during adsorption-electrophoresis.) Spermine (10 µM) and spermidine (100 µM) inhibit the basal activity of the highly purified enzyme 60-80% without affecting that assayed in the presence of calmodulin. Spermine stabilizes the enzyme against loss of activity when incubated at 30°C in the presence of metal chelators. Spermine appears to antagonize the stabilization provided by $MgCl_2$, suggesting that the polyamine may compete for a Mg^{++}-binding site as suggested in other systems. Interaction at such a Mg^{++} site could be related to the reduction of "basal" enzyme activity.

Endogenous protein inhibitors of the calcium-activated phosphodiesterase have been reported by other workers. We have purified two inhibitory proteins from bovine brain. One of these is heat-stable with a molecular weight of $\sim$ 12,000 on SDS-gels. It reduces the basal activity of the phosphodiesterase whether or not calcium is present and shifts to the right the dose-response curve for calmodulin activation. Many similarities between calmodulin and troponin C have been noted, and it is possible that regulatory elements for

the phosphodiesterase might exist as a complex similar to the troponin-tropomyosin complex found in skeletal muscle. We have found that purified troponin components are capable of modulating cGMP phosphodiesterase activity from bovine brain. In the presence of calcium, troponin C increased the activity of the enzyme, while in the absence of calcium, troponin I and troponin T inhibited phosphodiesterase activity. On the other hand, calmodulin from bovine brain could be substituted for troponin C in forming the troponin-tropomyosin complex and produced calcium-sensitive activation of actomyosin ATPase.

Work in many laboratories has shown that many mammalian tissues contain three major types of soluble phosphodiesterases which can be separated on the basis of their behavior on anion exchange chromatography. We have found this year that, in addition to these three enzymes, the rat liver supernatant contains a fourth form of phosphodiesterase. This has been separated and its properties compared with those of the other soluble phosphodiesterases. It has a high affinity for cGMP but is not activated by calcium plus calmodulin or by limited proteolysis. Its molecular weight is $\sim$ 325,000, much greater than that of the calcium-activated phosphodiesterase. Further investigation of the characteristics of this enzyme and its relationship to the other cyclic nucleotide phosphodiesterases is in progress.

2. Adenylate Cyclase

In the past year, our adenylate cyclase work has, for the most part, been directed toward the mechanism by which choleragen activates the enzyme and the role of ADP-ribosylation. We have also continued investigation of the interaction of choleragen with gangliosides, specifically ganglioside G_{M1} which serves as a cell surface receptor for the toxin. In earlier studies with liposomal model membranes, release of trapped glucose was used as an index of permeability changes, and it was established that these resulted specifically when the B subunits of choleragen interacted with liposomes containing G_{M1}. A continuation of these collaborative experiments showed that the binding of ^{125}I-choleragen to liposomes was rapid and not readily reversible. Half-saturation occurred with 10^{-10} M choleragen, and similar concentrations of unlabeled toxin blocked the binding of ^{125}I-choleragen. Binding was specific for liposomes containing G_{M1}; only small amounts of toxin were bound to liposomes containing G_{M2}, G_{A1}, or G_{D1b}. Choleragen protected the G_{M1} in liposomes from labeling by sequential exposure to galactose oxidase and NaB^3H_4; liposomal G_{D1b}, G_{D1a} and G_{M2} were protected to a lesser extent. Binding of choleragen also reduced labeling of the sialic acid moiety of liposomal G_{M1} by $NaIO_4$ and NaB^3H_4. In sum, the interaction of choleragen with G_{M1} present in liposomes had the same specificity and characteristics noted in our earlier studies with cell membranes. Other collaborative experiments have shown that choleragen, which contains five B or binding subunits per molecule, can act as a lectin, linking G_{M1} molecules in different cells or liposomes and thereby causing agglutination.

Escherichia coli heat-labile enteroxin, believed to be involved in the pathogenesis of "traveler's diarrhea," cross-reacts immunologically with antisera directed against primarily the B subunits of choleragen. We have shown that NCTC 2071 cells, which are deficient in gangliosides and lack G_{M1}, respond to

choleragen with an increase in intracellular cyclic AMP only after the cells have incorporated exogenous G_{M1}. These cells did not raise their cAMP content in response to E. coli heat-labile enterotoxin. Following the uptake of G_{M1}, the cells responded with an increase in intracellular cAMP. These and other experiments established that ganglioside G_{M1} functions as a cell surface "receptor" for the E. coli toxin as it does for choleragen. It had been known that adenylate cyclase activation by the toxin was NAD-dependent, and we reported last year that, like choleragen, the E. coli enterotoxin exhibits ADP-ribosyltransferase activity; it catalyzes the transfer of ADP-ribose from NAD to arginine, several guanidino compounds and proteins. We have now purified to apparent homogeneity an ADP-ribosyltransferase from turkey erythrocytes that activates adenylate cyclase in the presence of NAD and catalyzes the same model reactions with the same stereospecificity as do the two toxins, although it is clearly different in structure and kinetic characteristics. In our early studies, purified proteins, e.g., lysozyme, histones, were used as ADP-ribose acceptors and the products characterized as mono-ADP-ribosyl protein. We are now investigating the ADP-ribosylation of endogenous proteins in supernatant and particulate fractions of brain and thymus catalyzed by choleragen or by the erythrocyte transferase to determine factors that may influence the rate, extent and specificity of these reactions.

Others have suggested that choleragen activation of adenylate cyclase results from the ADP-ribosylation of a GTP-binding protein leading to decreased GTPase activity and thereby prolonging the life of a postulated GTP-adenylate cyclase complex which is catalytically active. Thus far, evidence in support of this hypothesis has been obtained only in studies of turkey erythrocyte membranes, where choleragen inhibits a catecholamine-stimulated GTPase. We have found this year that prostaglandins increase GTPase activity in human mononuclear cell membranes. Choleragen activated adenylate cyclase in these preparations but had no effect on GTPase activity, whether or not PGE_1 was present. In addition, we were unable to demonstrate any consistent association between the effects of several prostaglandins on GTPase and on adenylate cyclase. In a detailed investigation of the role of GTP in choleragen activation of adenylate cyclase from bovine brain, we have been able to establish separate distinct requirements for this nucleotide in the activation (ADP-ribosylation) process and in the expression of catalytic activity of the activated enzyme, as suggested by earlier work. We have further shown that the choleragen-activated enzyme is rapidly (to a large extent irreversibly) inactivated at 30°C and can be remarkably stabilized by GTP. For activation, stabilization and catalytic function GTP is more effective than ITP which is more effective than ATP.

3. Guanylate Cyclase

Last year we reported the extensive purification of soluble guanylate cyclase from rat liver. We have now achieved further purification using affinity chromatography on GTP-Sepharose. This enzyme has a pI of ca. 6.2 based on analytical polyacrylamide gel isoelectric focusing. Following an early step in purification, the enzyme requires for maximal catalytic activity addition of a fraction separated at this stage. Numerous attempts to purify this "activator" using standard methods for protein fraction have been unsuccessful. It now appears that it may be a small molecule(s) that can associate

with several proteins. Currently, efforts are directed toward further characterization of the kinetic properties of highly purified enzyme and the preparation of antibody to it.

4. Regulation of Cholesterol Metabolism in Mammalian Cells

It is generally recognized that the rate of cholesterol synthesis in many mammalian cells is controlled by the activity of hydroxymethylglutaryl Coenzyme A (HMGCoA) reductase, which is subject to negative feedback regulation by cholesterol. The possibility that other enzymes in the pathway of cholesterol synthesis might be subject to similar control has been investigated this year. It has been found that mevalonate kinase activity in cultured human fibroblasts is altered in parallel with HMGCoA reductase activity under several conditions, although the changes in kinase activity are of considerably lesser magnitude. For example, after incubation of normal fibroblasts in lipid-deficient serum, exposure to whole serum or to suspensions of 25-hydroxycholesterol plus cholesterol depressed mevalonate kinase activity by 50%, whereas HMGCoA reductase activity was decreased > 90%. In fibroblasts from a patient with homozygous familial hypercholesterolemia, serum low-density lipoprotein, as expected, did not decrease HMGCoA reductase activity nor did it alter mevalonate kinase. Both enzyme activities were, however, depressed by 25-hydroxycholesterol. Insulin, which we had previously shown increases HMGCoA reductase activity in fibroblasts, also increased kinase activity about 100%. Both mevalonate kinase and phosphomevalonate kinase were assayed in rat liver, and it was found that cholesterol feeding decreased the activity of these enzymes as well as that of HMGCoA reductase. It appears that changes in the activities of several enzymes may contribute to the physiological regulation of cholesterol biosynthesis. In concurrent studies, we are extending earlier observations on the effects of progesterone and glucocorticoids on cholesterol synthesis, esterification, and release using cultured human fibroblasts.

5. Suppression of Cell Proliferation by Anti-inflammatory Drugs

We had previously shown that nonsteroidal anti-inflammatory drugs inhibit proliferation of cultured hepatoma (HTC) cells and fibroblasts. Cells are inhibited in the G_1 phase of the cell cycle and, on removal of indomethacin, resume growth in synchrony with cell division occurring after about 24 hr. The drug is taken up rapidly and reversibly by HTC cells and retention of drug is not a factor in the delay in resumption of growth. This year work has focused on the mechanism of action of these drugs, particularly their effects on membrane function and oxidative processes. Even in concentrations that suppressed cell proliferation completely, the drugs did not inhibit protein or RNA synthesis. The rate of DNA synthesis decayed by a first-order process with a half-life ($t_{1/2}$) of 18 hr in HTC cells. This appeared to be due to accumulation of cells in the G_1 stage of the cycle rather than to impairment of DNA synthesis itself. Several membrane transport systems were studied. The drugs inhibited selectively the "A" system, but not the "L" system, of amino acid transport. The "A" system is Na^+- and energy-dependent and has an affinity for a broad range of amino acids. The "L" system does not require Na^+ ions or energy and has an affinity for most neutral amino acids. The rate of transport (influx) of aminisobutyric acid (AIB), a sub-

strate for the "A" system, decayed immediately upon addition of indomethacin and reached a minimum in 3 to 4 hr. After removal of the drug, transport was restored within 6 hr. All of the anti-inflammatory drugs tested inhibited the transport of AIB, but none affected the facilitative transport of amino-bicycloheptylcarboxylic acid (a substrate for the "L" system), uridine, thymidine, or deoxyglucose. No marked changes in ATP/ADP ratio were associated with the changes in amino acid fluxes. It appears that the anti-inflammatory drugs have a rather selective action on biochemical/biophysical processes in the cell. Their ability to inhibit cell proliferation in a reversible fashion makes them potentially useful experimental tools in studies of the cell cycle.

As a counterpart to studies with cultured cells, we are using as a model the carrageenan-induced pleurisy in rats. Carrageenan induces an intense but short-lived (24 hr) inflammation which is characterized by accumulation of fluid, neutrophils and, during resolution of the inflammation, monocytes. White cells accumulate in the pleural cavity in numbers far exceeding those in the circulation. In thymidine-^{3}H-labeling experiments, mature neutrophils from the bone marrow were the major source of white cells in the exudate. Treatment with anti-inflammatory drugs decreased the response to carrageenan in a dose-dependent manner. Doses of indomethacin that inhibited the accumulation of neutrophils by as much as 85% had no effect on the production or release of cells from bone marrow or lymphoid organs. In contrast, neither methotrexate, which greatly depressed production and number of bone marrow cells, nor dexamethasone, which reduced the number of lymphocytes by 80%, had a marked anti-inflammatory effect in the carrageenan model. The studies suggest that the anti-inflammatory drugs' primary action is in preventing the recruitment of cells from the blood stream, and current studies are focusing on the effect of the drugs on production of chemotactic factors and chemotactic activity of the neutrophils.

6. Role of Histamine in Gastric Secretion and Regulation of Histamine Metabolism

Histamine, through stimulation of H_2 receptors and activation of adenylate cyclase in the parietal cell, is a physiological mediator of gastric secretion. Little is known, however, about its storage, release, or inactivation in the gastric mucosa. We have approached these questions using highly purified cell fractions from dog and rat gastric mucosa prepared in collaboration with Drs. Andrew Soll and Morton Grossman, Wadsworth V. A. Hospital, Los Angeles. Histamine and histidine decarboxylase were localized in a single cell-type. In the dog, this cell has the morphological characteristics of a mast cell and lies in close proximity to the parietal cell. In the rat, this cell is similarly situated but has the characteristics of an enterochromaffin cell-like cell. The histamine-containing cells from dog and rat lack receptors for IgE and are resistant to the histamine-depleting action of compound 48/80. In contrast to histamine, serotonin (and DOPA decarboxylase) is localized in an enterochromaffin cell which contains glucagon. Histamine-N-methyltransferase was found at very high levels in a fraction of large cells which contains mostly parietal cells.

Histamine synthesis was investigated in the histamine-containing cells from gastric mucosa, purified mast cells from dog livers and rat peritoneal mast cells. In the presence of physiological concentrations of histidine, the rate of histamine synthesis by intact cells was 20-30 times greater than that with extracts of these cells. Measurement of histidine uptake and studies with amino acids that blocked uptake but not histamine synthesis by the cell extracts indicated that histidine transport may be a rate-limiting step in histamine synthesis in the intact cell. This, in addition to histidine decarboxylase, could be a site of regulation of histamine synthesis in vivo.

Histamine-N-methyltransferase (HNMT) is present in high levels in gastric mucosa and inhibition of this enzyme might be expected to amplify the effects of histamine released under physiological conditions. Last year, we reported that Dimaprit, an H_2 histamine receptor antagonist, was a moderately potent, noncompetitive inhibitor of the enzyme. A systematic study of analogs of Dimaprit and histamine has shown that several H_2 receptor agonists are non-competitive inhibitors of the enzyme. We have postulated that the enzyme possesses an inhibitory site with an affinity for imidazole or similar system with localized π electrons. One compound, SKF Compound 91488, which is a potent inhibitor reported to have no agonist activity, was found to be an effective inhibitor of HNMT in vivo. When administered in combination with aminoguanidine (an inhibitor of diamine oxidase), almost complete block of histamine metabolism was produced.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HLB 00603-05 CM

PERIOD COVERED
October 1, 1978 through September 30, 1979

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:	Vincent C. Manganiello	Medical Officer (Res.)	CM	NHLBI
OTHERS:	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI
	James C. Osborne, Jr.	Staff Fellow	MD	NHLBI
	Mary Ann Danello	Staff Fellow	CM	NHLBI
	Gordon J. Strewler	Senior Staff Fellow	LKEM	NHLBI

COOPERATING UNITS (if any)

Warren Strittmatter, Laboratory of Biochemical Pharmacology, NIMH, Drs. Brent Reed and M. Daniel Lane, Department of Physiological Chemistry, Johns Hopkins University School of Medicine, Baltimore, Maryland

LAB/BRANCH

Cellular Metabolism

SECTION

Cellular Regulatory Mechanisms

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	0.7	1.3

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Work with the calcium-dependent cGMP phosphodiesterase is currently focused on mechanisms for regulation of calmodulin activity. Cyclic nucleotide phosphodiesterases in several tissues have been studied to characterize their regulatory properties. As part of a continuing investigation of the effects of glucocorticoids, we are comparing phosphodiesterases in liver and hepatoma cells. In other studies, we have found that with the differentiation of 3T3-L1 fibroblasts into cells having morphological and biochemical characteristics of adipocytes is the appearance of a particulate phosphodiesterase which exhibits a high affinity for cAMP and cGMP.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To characterize the multiple forms of cyclic nucleotide phosphodiesterases that catalyze the degradation of cAMP and cGMP in mammalian tissues and their relationships to each other. To elucidate the mechanisms through which the activities of these enzymes are regulated, e.g., by hormones, ions, or proteolysis.

Methods Employed: Cyclic nucleotide phosphodiesterase activity is assayed by published methods modified in this laboratory. For purification of phosphodiesterases and regulatory proteins (e.g., calmodulin) ion exchange chromatography, gel filtration, preparative electrophoresis and affinity chromatography are used as needed.

Major Findings: Characterization of liver and HTC hepatoma cell phosphodiesterase. HTC cells contain at least three classes of phosphodiesterases, two of which exhibit lower K_m's for cGMP than for cAMP and one of which exhibits a lower K_m for cAMP. Incubation of cells with dexamethasone produces a decrease in the activities of the enzymes which preferentially hydrolyze cGMP. None of the HTC cell enzymes is activated by Ca^{++} and calmodulin. The cells contain calmodulin-like material and the failure to detect this enzymatic activity cannot be accounted for by protease activity or the presence of inhibitors.

Rat liver supernatant contains the same three general classes of phosphodiesterases found in the HTC hepatoma cells. In addition, the Ca^{++}- and calmodulin-sensitive enzyme is present. In order of elution from DEAE columns in a gradient of NaCl, these are referred to as EIa, EIb, EII, and EIII. EIa exhibits a low K_m for cGMP and is not activated by Ca^{++} and calmodulin; EIb also has a high affinity for cGMP and is activated by Ca^{++} and calmodulin. EII has a high affinity for cGMP, and the hydrolysis of cAMP by this enzyme is stimulated by cGMP; EIII has a high affinity for cAMP. A purified lysosomal protease from rat kidney activates EIb from liver and EIII from liver and HTC cells, without affecting EIa or EII. Chymotrypsin also activates EIb but inactivates EIa, EII, and EIII. The characteristics and possible interrelationships between EIa, EIb and EII will be studied further.

We have separated EIa and EIb from rat liver. The two enzymes possess different molecular weights and kinetic properites. Gel filtration chromatographyof the two enzymes compared to known standards on the same AcA34 column indicates the molecular weight of the calcium, calmodulin-activatable enzyme to be 140,000, while that of the other enzyme is 325,000. The former enzyme displays normal Michaelis-Menten kinetics with a K_m = 7 μM, while the latter enzyme displays anomalous kinetic behavior with a K_m = 1-2.5 μM.

Regulation of calmodulin activity. Trifluoperazine, an antipsychotic drug, has been reported to inhibit specifically activation of phosphodiesterase by Ca^{++} and calmodulin. In collaborative studies with J. C. Osborne, Jr. (MDB, NHLBI), we find that trifluoperazine, in a concentration-dependent

fashion, reduces the α-helical content and disrupts the tertiary structure of calmodulin. This effect of the drug seems independent of added calcium. These data cast doubt on the presumed specificity of the trifluoperazine inhibition of calmodulin activation of phosphodiesterase.

In preliminary studies with W. Strittmatter and colleagues in the Laboratory of Biochemical Pharmacology, NIMH, we found that calmodulin can serve as an acceptor for a protein carboxyl-methyltransferase. Effects of methylation on calmodulin activity and ability to bind Ca^{++} will be studied.

Regulation of phosphodiesterase activity in 3T3-L1 fibroblasts. 3T3-L1 fibroblasts, under the influence of isomethylbutylxanthine, dexamethasone and insulin accumulate triglyceride and take on the appearance of fat-laden "adipocytes." In collaborative studies with B. Reed and M. D. Lane (Johns Hopkins University School of Medicine), we found that, in general, cyclic AMP and cyclic GMP phosphodiesterase activities are similar in 3T3-L1 (preadipocytes) and 3T3-C2 ("control") fibroblasts. Although cAMP phosphodiesterase activity is present in particulate fractions (100,000 x g) isolated from homogenates of both lines, only the activity in the differentiated cell line is inhibited (60%) by 1 μM cGMP. Furthermore, during differentiation of the preadipocyte into the "adipocyte," there is a marked increase in high affinity cGMP and cAMP phosphodiesterase activities of the particulate fraction. As this type of phosphodiesterase activity appears in the particulate fraction, the inhibition of cAMP phosphodiesterase activity by cGMP increases. The 3T3-L1 cell line may serve as a useful model to investigate regulation of a particulate, high affinity phosphodiesterase which is found in rat adipocytes and is activated by insulin or hormones that increase cAMP.

Significance to Biomedical Research: 1) Knowledge of the properties of cyclic nucleotide phosphodiesterases and their regulation is necessary to understand the mechanisms for regulation of cGMP and cAMP content in cells and tissues such as cardiac muscle and lung.

2) Definition of the mechanism of regulation of the calmodulin-sensitive enzyme system by Ca^{++} and various regulatory proteins may facilitate understanding how Ca^{++} acts in other enzyme systems, e.g., adenylate cyclase.

Proposed Course: 1) Complete characterization of the calmodulin-sensitive enzyme system and its regulatory components; raise antibodies to the various components.

2) Determine subcellular localization of the system and its relationship with guanylate cyclase, protein kinase, etc.

3) Define interrelationships between the several phosphodiesterase systems.

4) Investigate further development of cyclic nucleotide phosphodiesterases during differentiation, and regulation of their activity by proteolysis.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HLB 00607-06 CM

PERIOD COVERED
October 1, 1978 through September 30, 1979

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 Manganiello	Medical Officer (Res.)	CM	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cellular Metabolism

SECTION
Cellular Regulatory Mechanisms

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Soluble rat liver guanylate cyclase purified with GTP-Mn^{2+} affinity chromatography had a pI of 6.1 to 6.3 in analytical polyacrylamide gel isoelectrofocusing. The purified enzyme was activated by nitrohemoglobin as well as by an endogenous activator, and maximally activated in the presence of both together. Work on purification of this activator and of a low molecular weight heat-stable factor that permits nitroprusside activation of the guanylate cyclase is in progress.

PHS-6040
(Rev. 10-76)

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 $MnCl_2$, 1 mM cGMP, 6 mM theophylline and Tris-Cl buffer, pH 7.4. [^{32}P]cGMP was purified through columns for radioassay. The cyclase from supernatant of tissue homogenates is purified using salt fractionation and ion exchange, gel and affinity chromatography.

Major Findings: Hepatic guanylate cyclase. The guanylate cyclase which had been purified 2000- to 5000-fold from rat liver supernatant was specifically bound to a GTP-affinity column equilibrated with $MnCl_2$. The enzyme eluted with NaCl had an isoelectric point of 6.1 to 6.3 in the analytical polyacrylamide gel (APAG) isoelectrofocusing. On analytical polyacrylamide gels, the purified enzyme exhibited two bands that stained with Coomassie blue, one of which was associated with guanylate cyclase activity.

This preparation was activated by nitrohemoglobin as well as by the activator fraction from rat liver that we have previously described. The effects of nitrohemoglobin, like those of the activator, were greater with lower concentrations of enzyme. Activation by nitrohemoglobin and activator (in maximally effective concentrations) was additive, however, consistent with other evidence that the activator from liver is not nitrohemoglobin or a related compound. Several other proteins were tested in concentrations of 50 to 1000 µg/ml. Cytochrome C, fumarase, lactic dehydrogenase, lipoxidase, and cytochrome C reductase increased activity 30 to 60% under conditions in which activator increased it more than 200%. Bovine serum albumin, glyoxal reductase, ferritin, peroxidase, and lactate dehydrogenase either had no effect or inhibited guanylate cyclase activity. Numerous attempts to purify the activator from rat liver supernatant using standard methods for protein fractionation (e.g., ion exchange chromatography, alkyl-, phenyl-, or Con A-Sepharose, hydroxylapaptite) have been unsuccessful. It was initially excluded from Biogel A 1.5 but after acetone extraction activator activity was retained in Biogel A 0.5 and eluted over a broad range associated with several protein peaks. Detergents either destroyed activity (Triton X-100, sodium taurodeoxycholate) or markedly reduced it (Lubrol PX). When subjected (without acetone treatment) to flatbed isoelectricfocusing (pH 3.5 to 10), activator was spread between pH 4 and 6 (associated with very little detectable protein). It appears now that activator may be a small molecule(s) that can associate relatively tightly with several proteins.

We reported last year that boiled rat liver supernatant contains a low molecular weight compound(s) in the presence of which the partially purified guanylate cyclase can be activated by nitroprusside. Using gel filtration, ion exchange chromatography, countercurrent fractionation and paper chromatography, we have extensively purified this material but have been unable to identify it, in large part, because of its instability. It is clearly

not ascorbate or glutathione or cysteine, compounds which can, to some extent, make possible the demonstration of nitroprusside activation. Based on its chromatographic behavior, failure to react detectably with reagents for functional groups (aldehyde, ketone, double bond, sulfhydryl, primary or secondary amine, amino acid), apparent lack of UV. absorption, and other characteristics, it appeared that the "nitroprusside factor" might be a polyol, perhaps with a carboxyl group. However, none of the compounds of this type that we have tried can replace it.

Significance to Biomedical Research: Although at present information concerning the metabolism and functions of cyclic GMP is limited, it appears that this nucleotide may be of especial importance in the development, physiology, and pathology of lung, vascular smooth muscle, and kidney.

Proposed Course: We shall further characterize the properties of pure guanylate cyclase and also purify the activators in order to study their interaction and to establish the mechanisms by which the guanylate cyclase is activated.

Publications:

Tsai, S.-C., Manganiello, V. C. and Vaughan, M.: Activation of kidney guanylate cyclase by cobalt. Arch. Biochem. Biophys. 189: 155-160, 1978.

Tsai, S.-C., Manganiello, V. C. and Vaughan, M.: Purification of guanylate cyclase from rat liver supernatant. J. Biol. Chem. 253: 8452-8457, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HLB 00614-02 CM

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Calcium- and Activator-dependent cGMP Phosphodiesterase from Bovine Brain: 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	PRAT Fellow	CM	NHLBI
	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI
OTHER:	Vincent C. Manganiello	Medical Officer (Res.)	CM	NHLBI

COOPERATING UNITS (if any)
Dr. Kincaid was in the Pharmacology/Toxicology Program, National Institute of General Medical Sciences, NIH

LAB/BRANCH
Cellular Metabolism

SECTION
Cellular Regulatory Mechanisms

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

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)

A calmodulin-dependent cyclic nucleotide phosphodiesterase from bovine brain supernatant has been purified by means of a new procedure, termed "sequential adsorption-electrophoresis." This method provides rapid and extensive purification (2000-3000 x) in high yield (30-40%) and may be applicable to other calmodulin-binding proteins. The basal enzyme activity is inhibited noncompetitively by physiologic concentrations of polyamines, while Ca^{++}-dependent activity is unaffected. Spermine stabilizes activity of purified enzyme and appears to antagonize the stabilization provided by $MgCl_2$. The polyamines may compete for a Mg-binding site related to both stability and "basal" activity.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To purify the Ca^{++}- and activator-dependent cGMP phosphodiesterase(s) from bovine brain and characterize the enzymatic and physical properties of the protein. To establish the role of various effectors including the Ca^{++}-dependent "activator" (calmodulin) and the low molecular weight inhibitor protein in the regulation of cellular diesterase activity.

Methods Employed: Extensive purification of cyclic nucleotide phosphodiesterase has been achieved through the development of a new procedure, "sequential adsorption-electrophoresis," which combines adsorption of the enzyme to calmodulin-Sepharose with direct electrophoretic elution and separation from other calmodulin-binding proteins. Prior fractionation of enzyme by gel filtration is also employed.

Major Findings: Calmodulin-dependent cyclic nucleotide phosphodiesterase from bovine brain has been purified to apparent homogeneity by the following 3-step method; High speed supernatant (60 min, 100,000 x g) is depleted of calmodulin by batchwise adsorption of the activator to anion exchange resin. The unbound fraction containing 95% of the phosphodiesterase activity is then used for preparative-scale gel filtration of Ultrogel AcA34. The peak fractions from this step ($\sim$ 65% of that applied) are concentrated by Ca^{++}-dependent adsorption to calmodulin-Sepharose.

After washing, the adsorbent is applied to the surface of a 6% polyacrylamide gel and eluted electrophoretically in the presence of EGTA. The eluted phosphodiesterase activity ($\sim$ 65% of that adsorbed to the affinity gel) is concentrated by readsorption to calmodulin-Sepharose which also removes electrophoretic impurities. The peak enzyme fractions yield one major band on SDS-gels (MW $\sim$ 58,000) and a lower molecular weight component ($\sim$ 20,000 daltons).

Major findings of this study include the following: Treatment of the supernatant as outlined above yields only one major form of cGMP phosphodiesterase activity. Previous observations indicating multiple forms of phosphodiesterase probably reflect from limited proteolysis and/or fractionation procedures which generate different forms. The purification procedure enriches the activity of the supernatant 2000- to 3000-fold with an overall yield of 30-40% and can be easily carried out in three days.

The purified enzyme is activated $\sim$ 6-fold by calmodulin in the presence of Ca^{++} and is not significantly affected by Ca^{++} alone. Activation by calmodulin depends upon substrate concentration being more pronounced at low concentrations ($\sim$ 1 μM) and less at higher levels ($\sim$ 100 μM). The activity toward cGMP is 5-10 times greater than that for cAMP at micromolar substrate concentration and both activities co-elute as a symmetrical peak during adsorption-electrophoresis.

The purified enzyme has been stable for eight months stored at -60°C in 20% glycerol, displays activity identical to the material not frozen (i.e.,

responsiveness to effectors and kinetic properties are not changed).

Findings not related strictly to the purification of phosphodiesterase include the following: The polyamines spermine (10 μM) and spermidine (100 μM) can inhibit the basal activity of the highly purified enzyme 60-80% without affecting that assayed in the presence of calmodulin. The inhibition by the polyamines is related to the alkyl chain length (i.e., spermine > spermidine > putrescine) and is reversible.

Spermine (10-100 μM) stabilizes the enzyme against loss of activity when incubated at 30°C in the presence of metal chelators. Spermine appears to antagonize the stabilization provided by 2 mM $MgCl_2$, suggesting that the polyamines may compete for a Mg^{++}-binding site as suggested in other systems. Interaction at such a Mg^{++} site may be related to the reduction of "basal" enzyme activity.

Significance to Biomedical Research: Numerous hormones, neurotransmitters and other humoral agents exert their effects on mammalian cells by altering the synthesis and/or degradation of cAMP and cGMP. The development of the new procedure, "sequential adsorption-electrophoresis," may prove useful as a general methodology for purification of proteins which interact with affinity adsorbents. Thus, defining the mechanisms for regulation of these processes is important for understanding many pathological states and devising rational therapeutic approaches.

Proposed Course: To define the physical and regulatory properties of the pure phosphodiesterase and to prepare antibodies to it that can be used to characterize further the enzyme and to probe its relationship with other phosphodiesterases. To compare the phosphodiesterase with other calmodulin-binding proteins, particularly in terms of their mechanisms and sites of interaction with the regulatory protein.

Publications:

Kincaid, R.L., Manganiello, V.C., and Vaughan, M.: Effects of spermine on activity and stability of calcium-dependent 3',5'-guanosine monophosphate phosphodiesterase. J. Biol. Chem. 254: 4970-4973, 1979.

Kincaid, R.L. and Vaughan, M.: Sequential adsorption-electrophoresis: A combined procedure used for purification of calcium-dependent cyclic nucleotide phosphodiesterase. Proc. Natl. Acad. Sci. USA, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HLB 00615-02 CM

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Calcium- and Activator-dependent cGMP Phosphodiesterase: Regulatory Components in Bovine Brain

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

PI:	Charles E. Odya	PRAT Fellow	CM	NHLBI
	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI
	Vincent C. Manganiello	Medical Officer (Res.)	CM	NHLBI

COOPERATING UNITS (if any)
Dr. Odya was in the Pharmacology/Toxicology Program, National Institute of General Medical Sciences, NIH

LAB/BRANCH
Cellular Metabolism

SECTION
Cellular Regulatory Mechanisms

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.85	0.85	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 activity of a specific cGMP phosphodiesterase is increased in the presence of calcium and a calcium-binding protein, calmodulin. We have purified from bovine brain the phosphodiesterase, the calcium-binding protein, and other factors that appear to be involved in the modulation of the activity of the enzyme in order to define the nature of their interactions.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To purify from bovine brain protein factors that appear to be involved in the modulation of the activity of the specific cGMP phosphodiesterase that is activated by calcium and calmodulin in order to define the nature of the interactions of the several components.

Methods Employed: Cyclic nucleotide phosphodiesterase was assayed by a published method modified in the laboratory. Calmodulin and the specific phosphodiesterase were purified using ion exchange chromatography, gel filtration, electrophoresis and affinity chromatography as needed. Purification of phosphodiesterase activators and inhibitors is described below.

Major Findings: Acetone powder of bovine brain was homogenized in 20 mM phosphate buffer, pH 7.0. Supernatant from a 10,000 x g x 60 min centrifugation of the homogenate was applied to DEAE-cellulose 23 equilibrated with 50 mM Tris-acetate buffer, pH 6.0, containing DTT, EGTA, and sodium azide. The solution passing through this column contained inhibitors of the Ca^{++}- and calmodulin-stimulated cGMP phosphodiesterase. Elution of the column with a sodium acetate gradient yielded two fractions that activate cGMP phosphodiesterase in a calcium-dependent manner. These fractions were purified to apparent homogeneity after chromatography on DEAE-Sephadex A-25 and Ultrogel AcA54. One of the calcium-dependent activator proteins is calmodulin; the other is probably the so-called S-100 protein. Inhibitory proteins were purified by two different procedures.

1) The initial eluate of the first anion-exchange column was applied to CM-cellulose 23 equilibrated with the same buffer. The inhibitors were retained on the column and eluted with a sodium acetate gradient. Fractions containing the inhibitors were pooled and then filtered with an Amicon ultrafiltration device equipped with a membrane that has a 100,000 MW cutoff. The inhibitors passed thru this filter as well as a 50,000 MW filter but were retained by a 5,000 MW filter. Gel filtration of the 5,000-50,000 MW fraction on Ultrogel AcA54 yielded two peaks of inhibitory activity that were then chromatographed on Sephadex G-50. Based on their elution from that column, the molecular weights of the inhibitors were about 20,000 and 12,000. Both inhibitors were further purified on a calmodulin-affinity column. They were bound to this column in a calcium-dependent manner and eluted with chelating agents.

2) The extract of bovine brain acetone powder was made 40% saturated with ammonium. The supernatant was heated at 90° for 5 min. The soluble extract was dialyzed against buffer containing 6 M urea and chromatographed sequentially on DEAE-Sephacel, CM-Sepharose and AcA44. The inhibitor was then applied to a column of calmodulin-Sepharose in 2 M urea with calcium and did not bind. It did bind to heparin-Sepharose and was eluted with NaCl. This material exhibited one major bond on 15% SDS gels (MW $\sim$ 12,000). The inhibitor reduces the basal activity of the phosphodiesterase, whether or not calcium shifts the dose-response curve for calmodulin activation to the right. Its effects can be reversed by calmodulin.

Significance to Biomedical Research: Regulation of cyclic nucleotide phosphodiesterase activity the mechanisms for which are still poorly understood is of major importance in the control of intracellular concentrations of cAMP and cGMP.

Proposed Course: To complete purification and characterization of the proteins that interfere with activation of phosphodiesterase by calmodulin and to define the nature of the interactions between the enzyme and its regulatory components.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HLB 00617-03 CM

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
The Mechanism and Action of Anti-inflammatory Drugs; Effects on Neutrophil Mobilization in Carrageenan-induced Inflammation.

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

PI:	Alvair P. Almeida	International Research Fellow	CM	NHLBI
	Barbara Bayer	Staff Fellow	CM	NHLBI
OTHER:	Michael A. Beaven	Pharmacologist	CM	NHLBI

COOPERATING UNITS (if any)
Dr. de Almeida is an International Research Fellow, Fogarty International Center, NIH. Dr. Bayer was the recepient of a National Research Service Individual Postdoctoral Award from the NHLBI, NIH, until June 1979.

LAB/BRANCH
Cellular Metabolism

SECTION
Cellular Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 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)

Carrageenan-induced inflammation in rat pleural cavity was used as a model to study how cellular activity is altered by anti-inflammatory drugs during the inflammatory response. Although at early stages of the response the pleural exudate contained a relatively high proportion of "mast cells" and high levels of intracellular histamine, there was no evidence from studies with antihistamine drugs and Compound 48/80 that histamine was involved in this reaction. At later stages (2-8 hr), neutrophils appeared in large numbers (1-2 x 10^8 cells) in the cavity and their number increased in the circulation. Thymidine-labelling experiments indicated that the cells were derived from bone marrow and were 2- to 3-days old. Treatment with nonsteroid anti-inflammatory drugs produced a marked decrease (60-75%) in the number of neutrophils appearing in the exudate without effecting the turnover or release of white cells in bone marrow and lymphoid organs. In contrast, methotrexate and dexamethasone were not anti-inflammatory unless given for 3 to 5 days, when the number of granulocytes and lymphocytes in tissue and circulation were decreased by more than 70%. The nonsteroid anti-inflammatory drugs appear, therefore, to inhibit the recruitment of white cells from the circulation.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Carrageenan-induced pleurisy in rat was chosen as a model to study the mechanism of action of the anti-inflammatory drugs. Studies by other workers (see Fed. Proc. 35: 2447, 1977) have shown that carrageenan will induce an "inflammatory reaction" in the pleural cavity. The reaction is characterized by an accumulation of an exudate which contains large numbers of white cells (predominantly neutrophils and at later stages monocytes), and the numbers of cells mobilized are markedly suppressed by the anti-inflammatory drugs.

In this program, the effect of anti-inflammatory drugs on the release of inflammatory mediators, such as histamine, serotonin and the prostaglandins, as well as chemotactic factors will be investigated. We shall attempt also to identify the source of white cells and specific forms of cellular activity inhibited by the anti-inflammatory drugs, e.g., chemotaxis, phagocytosis and recruitment of cells from the circulation.

Methods Employed: Inflammation was induced in Sprague-Dawley rats (200 g) by injection of 500 µg carrageenan into the pleural cavity. Rats were killed at the indicated times and the pleural fluid collected. Volume of the exudate and cell counts were determined as described previously. Spleen, thymus, lymph nodes and femur were removed, freed from adhering tissues and placed in 2 ml of phosphate-buffered saline (PBS). The organs were cut open by a scalpel, and in the case of femur the marrow was scraped entirely from the bone. The cells were collected in the PBS by teasing and squeezing the tissues with tweezers. The suspension was filtered through gauze. Tissue remnants were rinsed several times to ensure quantitative extraction of cells. Differential cell counts were determined by use of the Cytocentrifuge and differential staining of the slides. Blood cell counts were obtained from air-dried smears of blood taken by cardiac puncture. Anti-inflammatory drugs were administered orally as suspensions in cornstarch gel. Other drugs and labeled compounds were injected as saline solutions by the routes indicated. Histamine and serotonin were assayed by the enzymatic isotopic techniques described in previous annual reports.

Major Findings: Time Course of Response to Carrageenan: Within 1 hr, small amounts of a viscous white fluid (0.1-0.2 ml) had collected in the pleural cavity. The exudate contained a large number of mast cells (15%) but no disrupted mast cells could be seen microscopically. After 60 min, increasing numbers of neutrophils appeared in the exudate, and by 4 hr the exudate (1-2 ml) consisted almost entirely of neutrophils (< 95%). The peak response occurred at about 8 hr, at which time monocytes were also observed in the exudate. Thereafter, the volume and numbers of cells decreased, and by 24 hr the inflammation had largely subsided.

Neutrophil counts at the peak response ranged from 72 to 180 x 10^6 cells/ml. Although there was a modest increase in the numbers of circulating neutrophils (27-65%), the total number of neutrophils in the chest cavity far exceeded that in the circulation. The neutrophils, therefore, arose

from reservoirs other than blood.

Thymidine-labelling Experiments. Thymidine-labelling studies indicated that the neutrophils were derived from the bone marrow and were mature cells (i.e. more than 2-days old). Labeled neutrophils, for example, appeared in pleural cavity only when thymidine-^{3}H was injected at 2-3 days previously. At this time, the specific activities (dpm/cell) of the exudate and bone marrow cells were identical and were the highest in the body.

Role of Histamine in the Inflammatory Response. Our earlier studies with inflammation induced by scalding of the rat paw (Eur. J. Pharmacol. 27: 305-312, 1974) showed a close correlation between the intensity of the reaction and histamine release. Studies with antihistaminic drugs and Compound 48/80 also provided evidence that histamine release played an essential role in the initial stages of the response.

There was no evidence for such a role for histamine in carrageenan-induced pleurisy, although the exudate contained large amounts of histamine (9-21 µg/ml). The mast cell appeared to be the major or sole repository for the amine. No increase in the levels of extracellular histamine (50-80 ng/ml) or decrease in the total number of mast cells or total histamine content of the exudate was observed at any time. Depletion of histamine stores by injection of small doses of Compound 48/80 (0.1 mg/kg) directly into the pleural cavity had no effect on the response to carrageenan (Table 1). Previous attempts to deplete the stores by intraperitoneal injection of Compound 48/80 were unsuccessful (Table 1). Pretreatment with H_1 and H_2 histamine antagonists in doses that have been shown to block receptors in vivo did not diminish the response. In contrast, the edema produced by intrapleural injection of Compound 48/80 was largely blocked by the inhibitors (Table 2).

Studies with Anti-inflammatory and Other Drugs. The accumulation of fluid was markedly reduced by pretreatment with aspirin, (ED_{50} 40 mg/kg), phenylbutazone (ED_{50} 100 mg/kg) or indomethacin, (ED_{50} 2.5 mg/kg) in a dose-dependent manner. The infiltration of neutrophils (and to a lesser extent monocytes) was suppressed also. After chronic administration of indomethacin (10 mg/kg, b.i.d. for 3 days), the reaction was suppressed even further (Table 3).

Indomethacin in single or repeated doses had no effect on the number of white cells (Table 3) or on incorporation of labeled thymidine into cellular DNA (data not shown), in bone marrow, spleen, thymus, lingual lymph glands and blood. In contrast, the number of bone marrow cells was markedly diminished after chronic administration of methotrexate as were the number of thymus and spleen lymphocyte cells after treatment with dexamethasone. However, neither of these two drugs had a marked anti-inflammatory action. Only after prolonged treatment (5 days) with methotrexate. When the granulocyte content of bone marrow was reduced by 70%, was a reduction in the reaction to carrageenan apparent (Table 3).

Significance to Biomedical Research: Carrageenan-induced edema appears to be a useful model to study the effects of various drugs on white cell recruitment. With this model we have shown that 1) indomethacin and other nonsteroidal anti-inflammatory drugs inhibit the recruitment of white cells during an inflammatory reaction without affecting the dynamics of white cell production and release in bone marrow nad other organs and 2) these drugs have quite different actions from those of methotrexate and dexamethasone.

Proposed Course: Subsequent studies will investigate whether the suppression of neutrophil accumulation is due to an effect of the drugs on the production of chemotactic factor(s) or on the ability of the cells to respond to chemotactic factors.

Publications: None

Table 1. Effect of Compound 48/80, Given by Intraperitoneal or Intrapleural Injection, on Exudate Formation Induced by Intrapleural Injection of Carrageenan

Exp.	Treatment*	(n)	Pleural Exudate			Skin
			Volume (ml)	White cells ($\div 10^6$)	Histamine (μg)	Histamine (μg/g)
Cpd. 48/80(1 mg/kg) (intraperitoneal inj.)						
1	Carrageenan	(9)	1.4 ± 0.1	--	20.9 ± 1.9	17.4 ± 1.2
	Cpd. 48/80-Carrageenan	(9)	1.0 ± 0.1	--	16.9 ± 7.2	7.2 ± 1.0
2	Carrageenan	(9)	1.8 ± 0.2	146 ± 19	29.3 ± 5.5	--
	Cpd. 48/80-Carrageenan	(8)	1.4 ± 0.4	277 ± 44	17.1 ± 1.6	--
Cpd. 48/80(0.05 mg/kg) (intrapleural inj.)						
3	Carrageenan	(6)	1.1 ± 0.2	101 ± 8	1.4 ± 0.4	--
	Cpd. 48/80-Carrageenan	(6)	0.7 ± 0.1	100 ± 6**	0.5 ± 0.2	--
	Cpd. 48/80	(6)	0.4 ± 0.1	33 ± 3**	--	--

Values are mean ± SEM

* Cpd. 48/80 was administered 24 hr before the injection of carrageenan.

** No mast cells were visible in these exudates. The exudates in the Cpd. 48/80-carrageenan-treated group were mostly neutrophils and in the Cpd. 48/80-treated group, eosinophils.

Table 2. Effect of Antihistamine Compounds on Formation of Pleural Exudate in Response to Injection of Carrageenan or Compound 48/80

Pretreatment	(n)	Carrageenan Injected Rats		Cpd. 48/80-Injected Rats	
		Volume (ml)	White Cells ($\div 10^6$)	Volume (ml)	White Cells ($\div 10^6$)
None (control)	(6)	1.0 ± 0.1	89 ± 8	0.33 ± 0.08	< 2
Mepyramine (20 mg/kg)	(6)	0.8 ± 0.1	81 ± 5	0.29 ± 0.05	< 2
Metiamide (50 mg/kg)	(6)	0.9 ± 0.1	79 ± 6	0.31 ± 0.04	< 2
Mepyramine (mg/hg)+	(6)	0.9 ± 0.1	93 ± 6	0.12 ± 0.03	< 2

Values are mean ± SEM. Drugs were administered by intraperitoneal injection 30 min before the intrapleural injection of carrageenan (500 µg) or Compound 48/80 (50 µg/kg). The rats were killed 4 hr after the injection of carrageenan and 1 hr after the injection of Compound 48/80.

Table 3. Effect of Anti-inflammatory and Immunosuppressant Drugs on White Cell Populations of Pleural Exudate, Bone Marrow and Lymphoid Organs

Treatment		n	Change in White Cell Count			
			Pleural fluid	Bone marrow	Thymus	Spleen
			% of Control			
Indomethacin	10 µg/kg, p.o.	5	4 ± 0.2	100 ± 9	89 ± 12	138 ± 17
Dexamethasone	500 µg/kg, s.c., 3 days	7	72 ± 13	102 ± 11	6 ± 0.8	36 ± 6
Methotrexate	", i.p., 3 days	6	58 ± 9	43 ± 11	80 ± 12	83 ± 13
"	", ", 5 days	5	12 ± 4	27 ± 7	-	-

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HLB 00619-03 CM

PERIOD COVERED
October 1, 1978 through September 30, 1979

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 Pharmacologist CM NHLBI

COOPERATING UNITS (if any)
Drs. Andrew H. Soll and Morton I. Grossman, Center for Ulcer Research and Education, Wadsworth V.A. Hospital, Los Angeles, Calif.

LAB/BRANCH
Cellular Metabolism

SECTION
Cellular Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.5	0.1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

In collaborative studies with the Center for Research Education, V.A. Hospital, Los Angeles, the histamine-containing cell in dog stomach has been identified as a small mast cell which contains a specific histidine decarboxylase of low activity and has a histamine content of around 3 pg/cell. These cells are found exclusively in the lamina propria and lie in close proximity to the parietal cell. The cell exhibits the characteristic metachromasia upon staining with toluidine blue. Unlike the mast cell, it is resistant to the histamine-depleting action of compound 48/80. Fractionation of other cells in the dog mucosa by centrifugal elutriation has shown that serotonin and DOPA decarboxylase are present in enterochromaffin cells. In rat gastric mucosa in contrast to dog, histamine resides in an enterochromaffin-like (ECL) cell which contains abundant histidine decarboxylase activity and has no receptor for IgE.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The finding that the histamine H_2 receptor antagonists block gastric secretion in a variety of species provided convincing evidence that histamine has an essential role in gastric secretion. Its precise role in gastric secretion, however, is still unclear. The relationship of histamine to other mediators of gastric secretion, gastrin and acetylcholine, is poorly understood; nor is it known how histamine is released or how it reaches the acid-secreting parietal cell. The successful separation and identification of the different cell populations in dog gastric mucosa in collaboration with Soll and Grossman at the Center for Ulcer Research and Education (CURE), Wadsworth V.A. Hospital, Los Angeles, was described in last year's annual report. This approach enabled us to identify the "histamine-containing cell" in the gastric mucosa and to determine the cellular location of the enzymes responsible for histamine synthesis and metabolism. In this report, we described further studies which compare and contrast histamine-containing cells in dog and rat mucosa with mast cells in dog liver and rat peritoneum.

Methods Employed: Gastric mucosa and liver were treated sequentially with a collagenase preparation, EDTA, and then a second time with collagenase. At all stages, cell viability was monitored by microscopy and dye exclusion tests. The cells were harvested from the disrupted mucosa by filtration, washed, and subjected to a preliminary separation in a Sorvall zonal rotor using 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 elutirator. Gland preparations (isolated glandular crypts) were prepared by partial digestion with collagenase and centrifugation through Ficoll solution. Rat peritoneal cells were prepared as described in another report. 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, histidine and DOPA decarboxylase activity, and histamine-N-methyltransferase (HNMT) and diamine oxidase activities using the microassays developed in this laboratory. These assays have been described in previous annual reports, although further refinement of some procedures was necessary because of the small size of the samples. Assays were also performed by the principal investigator on fresh samples at CURE.

IgE binding was studied by procedures devised by Dr. Henry Metzger (NIAMDD), who supplied ^{125}I-labeled rat myeloma protein for these studies.

Major Findings: Isolation and Properties of the Histamine-containing Cells in Dog Liver and Fundic Mucosa. Profiles obtained by separation of different cell fractions first by elutriation and then by density gradient separations produced a single sharp peak of histamine for both dog liver and mucosa. In both tissues, the highest histamine concentration was found in cells with a mean density of 1.080 and a size of 9 μM. Mast cells constituted more than 80% of the cells in their fractions. The average histamine

content was 2.1 pg/cell for dog mucosa (Table 1) and 1.8 pg/cell for liver. In preparations in which toluidine-stained sections were available for the entire gradient, there was a close correlation (r = 0.94) between mast cell count and histamine content. Electron micrographs of these fractions confirmed that a high proportion of the cells possessed the typical morphology of mast cells, with characteristic variable granular structure. Ninety-three percent of the cells were viable as indicated by trypan blue exclusion.

Measurement of DNA as well as histamine at all stages of purification of the dog mucosa cells provided additional information (Table 1). The histamine content of the unfractionated mucosal cells was 69% of the histamine content in intact mucosa (Table 1). Seventy to 80% of the histamine loaded into the elutriator rotor and 85 to 90% of the histamine loaded into the density gradients were recovered in the eluted cell fractions. On the assumption that each mast cell contained 2.1 pg histamine, it was calculated that the mucosa contains 3×10^7 mast cells/g or about one mast cell per 3 parietal cells.

In morphological studies of intact mucosa, the mast cells were found in the lamina propria in close proximity to the basal surface of the parietal cells. Most parietal cells appeared to be close to or abut onto a mast cell. In surveying large numbers of mast cells both by light and electron microscopy, we failed to find mast cells on the glandular side of the basement membrane of the epithelium. Preparations of gastric glands, which were separated from the basement membrane and lamina propria by the enzyme treatment, contained less than 20% of the histamine found in either the whole mucosa or unfractionated mucosal cell suspension (Table 1).

Preliminary studies with the mast cells have indicated that histamine is released by ascaris antigen and Compound 48/80 in a dose-dependent manner from dog liver mast cell but not from the mucosal cells. The resistance of the mucosal cell to the liberating action of Compound 48/80 was also evident in studies with both rat peritoneal cells and the dog mucosal cell (Table 2). The data suggest that we do not yet have optimal conditions for release and further studies are necessary.

Separation of Histamine- and Serotonin-Containing Cells in Dog and Rat Gastric Mucosa: In both rat and dog cells, the histamine- and serotonin-containing cells were readily separated by density gradient elutriation. The histamine content of the most enriched (85%) preparation was 2.9 pg/cell for the dog mucosal cell compared to 16 pg/cell for purified rat peritoneal mast cells or 1-1.5 pg/cell for purified human basophils. For the serotonin-containing cells, the serotonin content has ranged from 0.3-0.6 pg/cell.

The histamine-containing cell in the rat mucosa was, however, quite different from that in dog mucosa. It lacked the typical granular appearance of the mast cell and had the staining characteristics of an enterochromaffin-like (ECL) cell described by other authors (Thunberg, Exp. Cell Res. 47: 108-115, 1967; Aures et al., Life Sci. 7: 1147-1153, 1968). These cells like the mast cell in dog mucosa lie in close proximity to the parietal cell.

Their histamine content has ranged from 1-1.9 pg/cell.

Chromaffin cells account for much of the serotonin content (0.3-0.6 μg/cell) in dog and rat mucosa, although some serotonin is also present in mast cells of rat. DOPA decarboxylase activity is high in the chromaffin cell.

Studies with the rat (IgE) myeloma protein indicated that the rat mucosal histamine cell lacked significant numbers (< 2,000 sites) of binding sites for the F_c IgE fragment. Peritoneal mast cells taken from the same animals contained from 240,000-560,000 receptors/cell (mean 420,000, n = 6), a value that was comparable to that (330,000) obtained by Metzger and associates (Nature 264: 548, 1976).

Location of Histidine Decarboxylase, Histamine-N-methyltransferase and Diamine Oxidase in Gastric Mucosa. Histidine decarboxylase activity was confined to the histamine-containing cells and was especially high in the rat histamine cells. The profile of HNMT activity generally paralleled that of parietal cell, although some activity appeared to be present in other cells. The HNMT activity in the parietal cell fraction was the highest that we have observed in dog or rat tissues. The histamine-containing cells were virtually devoid of HNMT activity. Diamine oxidase activity could not be detected in any cell fraction from dog mucosa. In rat, some diamine oxidase activity was present and appeared to be confined to cells of high density. These cells have not been identified.

Significance to Biomedical Research: The studies with isolated gastric mucosal cells have led to the identification of the cellular source of histamine and histidine decarboxylase in gastric mucosa. These cells appear to be in close proximity to the parietal cell. Previous studies by Soll have shown that, although the parietal cell has receptors for gastrin and acetylcholine, the presence of small amounts (10^{-7} M) of histamine is required for maximal stimulation of the parietal cell by these agents.

The other finding to which we attached particular significance is the high HNMT activity associated with the parietal cell. This enzyme presumably has a key role in inactivating histamine in the mucosa and provides another point for pharmacological intervention in studies of gastric secretion.

Proposed Course: Future studies will focus on possible release mechanisms for the histamine stores in the gastric mucosal cells, particularly their responsiveness to gastrin and cholinergic agents. The possibility that histidine decarboxylase activity is induced in these cells by gastrin will also be examined. More detailed biochemical examination of these cells (e.g., activation of adenylate cyclase) will follow once the agents inducing histamine release have been identified.

Publications: Author: Beaven, M.A.: Histamine: Its Role in Physiological and Pathological Processes. Monographs in Allergy, Vol. 13 Basel, S. Karger, 1978, 114 pp.

Shaff, R.E. and Beaven, M.A.: Increased sensitivity of the enzymatic isotopic assay of histamine: Measurement of histamine in plasma and serum. Anal. Biochem. 94: 425-430, 1979.

Soll, A.H., Lewin, K., and Beaven, M.A.: Isolation of histamine containing cells from canine fundic mucosa. Gastroenterology, in press.

Table 1: Histamine and DNA content of different fractions at various stages of purification of the dog mucosal mast cell

	Histamine		
	ng/µg DNA	pg/cell	µg/g tissue
1. Whole stripped mucosa	18.5 ± 6.1(5)	-	77 ± 20
2. Isolated gastric glands	3.0 ± 0.9(3)	-	-
3. Unenriched mucosal cells	12.8 ± 2.9(8)	0.11 ± 0.02(8)	-
4. Elutriator: histamine peak	-	0.54 ± 0.10(8)	-
5. Density gradient: histamine peak	-	2.05 ± 0.33(3)	-

Histamine content is the mean ± SE for the number of preparations indicated in parenthesis.

Table 2: Effect of Compound 48/80 on dog gastric mucosa and rat peritoneal mast cell

Treatment		Histamine Content in Rat Peritoneal Mast Cell pg/cell	Histamine Content in Dog Gastric Mucosal Histamine-containing Cell pg/cell
Control	0 min	7.72	1.01
	30 min	6.88	0.81
48/80 (1 µg/ml)	0 min	7.72	0.96
	30 min	1.59	0.90

Cells were incubated for 30 min in Earles' balanced salt solution (0.2% BSA, 10 mM HEPES, gassed with 95% O_2/5% CO_2). Cellular content of histamine was determined at the start and end of the incubation in the presence and absence of 1 µg/ml of Compound 48/80.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HLB 00620-02 CM

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Regulation of Histamine Synthesis and Metabolism 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	Pharmacologist	CM	NHLBI

COOPERATING UNITS (if any)
Andrew Soll, Center for Ulcer Research and Education, Wadsworth V.A. Hospital, Los Angeles, Calif.

LAB/BRANCH
Cellular Metabolism

SECTION
Cellular Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.3	0.3

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Studies with purified mast cells from rat tissues have shown that in the presence of physiological concentrations of histidine the rate of histamine synthesis is much greater in the intact cell than that in extracts of these cells and that histidine uptake is an essential component of the histamine synthetic pathway in the intact cell. Histamine synthesis is suppressed by inhibitors both of histidine transport and histidine decarboxylase. In many species histamine-N-methyltransferase is a key enzyme in the inactivation of histamine. We suggested that the enzyme possesses an inhibitory site with an affinity for histamine and a variety of H_2 histamine receptor agonists and on this basis looked for new inhibitors of the enzyme. One compound, SKF 91488, was found to be a particularly strong noncompetitive inhibitor (Ki $9^{-} \times 10^{-7}$M) of the enzyme in vitro and in vivo.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Our studies on the source of histamine in gastric mucosa raised several questions related to histamine metabolism. The histidine decarboxylase activity in the mucosal mast cells in dog was insufficient to account for the high histamine level in these cells. The paradoxically low and high HNMT activity associated with the parietal cell also suggested that inhibitors of HNMT would be particularly useful in studies of the role of histamine in gastric secretion. This program is directed to studies of histamine synthesis in intact cell systems and of inhibitors of HNMT. Previously described inhibitors were unsuitable in that they are only weakly active in vivo or have pharmacological actions of their own. Because the enzyme is inhibited by histamine in concentrations of 10^{-5} M + greater and H_2 histamine receptor agonists inhibit the enzyme in a noncompetitive manner we postulated that HNMT possessed an inhibitory site with a selective affinity for the imidazole ring or similar systems with delocalized π electrons. On this basis we tested a wide range of compounds and activity and found one compound, SKF 91488, to be a potent inhibitor in vitro.

In this report we shall describe our studies on the efficacy of this compound as an inhibitor of histamine metabolism in vivo. We shall also report the finding that in intact mast cells the rate of histamine synthesis 10-20 times greater than that in extracts of these cells and will show that histidine uptake is an essential component of the histamine synthetic pathway.

Methods Employed: Histamine-N-methyltransferase was obtained from two sources, guinea pig brain and rat kidney. The enzyme was prepared from soluble extracts of these tissues by ammonium sulfate fractionation procedures. Enzyme activity was assayed by measurement of the rate of formation of ^{14}C-methylhistamine from 50 µM histamine in the presence of S-adenosyl-L-methionine (^{14}C-methyl). These procedures have been described in previous reports. For kinetic studies with histamine-N-methyltransferase, the concentration of histamine was varied by the addition of unlabeled compound. The concentration of S-adenosyl-L-methionine (^{14}C-methyl) (5 x 10^{-6} M) was unchanged. To determine the "apparent" K_i value for the inhibitors, Dixon plots were prepared with the aid of a Cannon SX computer.

For in vivo studies with the HNMT inhibitor, 91488, drugs were given by intraperitoneal injection and β-^{3}H-histamine administered intravenously in tracer amounts (1.5 µli/mouse). The animals were placed in glan metabolic cages and the urine collected. The urine was assayed for labeled histamine and metabolites by chromatographic and isotope dilution procedures described in other publications from this laboratory (Beaven et al., Europ. J. Pharmacol. 29: 138, 1974). Endogenous histamine levels were determined by the enzymatic assay.

Intact mast cells were obtained from male, Sprague Dawley rats (180 to 300 g). After decapitation, 10 ml of Hanks' balanced salt solution were injected into the abdominal cavity, and a peritoneal cell suspension was

obtained by standard procedures. The cells were fractionated by elutriation on a Beckman elutriator rotor. Cell counts and size analysis were performed using a Particle Data Counter.

For the assay of histamine and enzyme activities, 1 ml aliquots of the cell suspensions were centrifuged at 100 g for 10 min and the supernatant fluid removed by aspiration. The cell pellets were resuspended in Gey's salt solution containing HEPES buffer (100 mM, pH 7.4) to give 10^6 cells/200 µl. Histamine was assayed by the enzymatic isotopic assay. Histidine and DOPA decarboxylase activity was assayed in 5- and 10-µl aliquots of the undiluted preparations by a microprocedure in which $^{14}CO_2$ release from ^{14}C-carboxyl-labeled L-amino acid was measured. The specificity of the assay for histidine decarboxylase activity was verified by incubating the samples with β-^3H-(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 removed and assayed for ^3H-histamine by isotope dilution derivative analysis. For these assays, the concentration of amino acid was 1.25×10^{-4} M, unless noted otherwise.

Uptake of β-^3H-L-histidine was measured by incubation of 10^6 mast cells with 100 nCi (10 pmoles) β-^3H-L-histidine in 100 µl Gey's (+ 50 mM HEPES, pH 7.4) medium for 10 min at 37°C and, in separate samples, at 0°C. The cells were deposited by centrifuging the samples through dibutylphthalate in a Microfuge (Beckman Instrument Corp.) for 1 min. The difference in ^3H content of the pellet incubated at 37°C with that incubated at 0°C was designated "uptake." The percent decrease of this "uptake" in the presence of drugs and amino acid was calculated.

Major Findings: Inhibition of histamine metabolism in vivo by SKF 91488. The major urinary metabolites of β-^3H-histamine were methylimidazole acetic acid and methylhistamine (Table 1). Imidazole acetic and the ribosyl conjugate of imidazole acetic acid constituted less than 30% and ^3H-histamine less than 10% of the ^3H label in the urine (Table 1). Blockade of diamine oxidase by pretreatment with aminoguanidine resulted in a small increase in the excretion of unchanged ^3H-histamine (Table 1). Administration of Compound 91488, in addition, led to a much larger increase with 37% of the label appearing as ^3H-histamine after 50 mg/kg Compound 91488 and progressively greater amounts with higher doses of Compound 91488 (Table 1). With the highest dose of Compound 91488 tested, 1000 mg/kg, most (> 85%) of the label appeared as unchanged histamine (Table 1). The increase in histamine excretion was accompanied by a decrease in the proportion of methylhistamine and methylimidazole acetic acid.

Histamine-N-methyltransferase activity in kidneys of rats given 250 mg/kg drug was inhibited completely when assayed in vitro. In contrast, the drug did not appear to enter the CNS in appreciable quantities. Histamine-N-methyltransferase activity and histamine levels in brain were unaltered even by large doses of drug. Since partial inhibition of the enzyme occurs with 10^{-7} M drug actual drug concentration in the brain was probably less

than 10^{-6} M.

The drug was excreted in urine. Urine, for example, collected from rats given 250 mg/kg SKF 91488 completely inhibited histamine-N-methyltransferase activity in vitro. After serial dilution (1:10, 1:50, 1:100 and 1:500) of the urine specimens and comparison of the inhibitory activity with solutions of known drug concentration, the 6-h urine collections from rats given 10 and 250 mg/kg of drug (n = 4 for each dose) were calculated to contain from 47 to 62% of administered drug.

No gross behavioral effects of drug were noted in mice or rats. In mice given 1000 mg/kg doses of drug, no cyanosis, lacrimation, respiratory difficulties, ataxia, or other toxic signs were noted. The mice maintained normal eating and grooming behavior.

Histidine uptake and histamine synthesis in the intact rat mast cells. Separation of rat peritoneal cells into different fractions by elutriation yielded a single peak of histamine in which mast cells comprise 80 to 98% of the cells. The histamine content of these cells (mean ± SEM) was 16 ± 1.8 pg/mast cell (n = 5). The distribution of histidine decarboxylase activity paralleled closely that of histamine, although the rate of histamine formation by intact cells was much greater than that of soluble extracts of these cells. The magnitude of this difference was similar when histdine 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 by α-methylDOPA and was not associated with detectable decarboxylation of L-DOPA.

The kinetics of decarboxylation in intact cells differed in several respects from that of sonicated cell extracts. The rate of $^{14}CO_2$ release declined after 30 min and was proportional to the number of mast cells with concentrations up to 5×10^4 cells/20 µl. With the extracts, $^{14}CO_2$ release was linear with time for up to 90 min and for concentrations of up to 2×10^5 cells/20 µl. The relationship of substrate concentration and enzyme activity showed that the apparent K_m was lower for the intact cell (0.5×10^{-4} M) than that of the soluble extract (5×10^{-4} M).

The contribution of histidine transport to the synthetic activity of the intact cell was investigated by examining the effects of amino acids which might compete with the uptake of L-histidine. L-Tryptophan and α-methylDOPA inhibited decarboxylation as well as uptake of L-histidine in the intact cell without inhibiting the histidine decarboxylase activity of extracts. The decarboxylase inhibitors, Brocresine and α-hydrazino-histidine, selectively inhibited decarboxylase activity without interfering with L-histidine uptake, whereas α-methylhistidine partially inhibited both. Other compounds which inhibited decarboxylation in intact cells but not that in extracts included β-2-thienylalanine and 2-fluoro-L-histidine. The inhibition was dose-dependent with LD_{50} values of 3 x and 5 x 10^{-4} M respectively.

The marked reduction in histidine decarboxylation upon sonication of cells was observed with purified mast cells and mixed cell suspensions from the peritoneal and pleural cavity of the rat. The reduction was the same whether the cells were disrupted by freezing and thawing or sonication. Such procedures did not appear to affect the enzyme directly when tested on a partially purified preparation of histidine decarboxylase from rat stomach.

Significance to Biomedical Research: The finding that SKF 91488 is an effective inhibitor of HNMT in vitro and in vivo will facilitate studies of the participation of histamine in physiological and pathological reactions. The studies demonstrate that histamine inactivation can be blocked entirely by administration of aminoguanidine and SKF 91488. The studies with most cells indicate that for maximum histamine synthetic activity the intact cell is required. The inhibition of both uptake and decarboxylation of histidine by tryptophan and other amino acids suggest that histidine transport is an important component of the histamine synthesis pathway in the intact cell. Thus histamine synthesis in the intact cell may be blocked by inhibiting histidine transport or by inhibiting the enzyme directly, and this provides another possible mode of inhibiting histamine synthesis by therapeutic agents.

Proposed Course: Studies of histamine synthesis will be continued with intact mast cells from dog liver and mucosa. Factors influencing histidine uptake will be studied in more detail.

Publications:

Baylin, S.B., Weisburger, W.R., Eggleston, J.C., Mendelsohn, G., Beaven, M.A., Abeloff, M.D. and Ettinger, D.S.: Variable content of histaminase, L-DOPA decarboxylase and calcitonin in small cell carcinoma of the lung--biological and clinical implications. New Engl. J. Med. 299: 105-110, 1978.

Beaven, M.A. and Shaff, R.E.: New inhibitors of histamine-N-methyltransferase. Biochem. Pharmacol. 28: 183-188, 1979.

Baylin, S.B., Hsu, T.-H., Stevens, S. and Beaven, M.A.: The effects of L-DOPA on in vitro and in vivo calcitonin release from medullary thyroid carcinoma. J. Clin. Endocrinol. Metab. 48: 408-414, 1979.

Ottesen, E.A., Paranjape, R., Thirwengadam, K.V., Trepathy, S.P., Beaven, M.A. and Neva, F.A.: Specific allergic sensitization to filial antigens in the tropical eosinophilia syndrome. Lancet I: 1158-1161, 1979.

TABLE 1

Effect of SKF Compound 91488 and aminoguanidine (Ag) on urinary excretion of labeled histamine and histamine metabolites in mouse

Treatment	(n)	Percent of label in urine excreted as:		
		Histamine[a]	Methyl-histamine[a,b]	Acid metabolites[b]
None	(4)	8 ± 2	34 ± 2	57 ± 3[c]
Aminoguanidine (Ag)	(3)	22 ± 1	38 ± 5	41 ± 6
Ag. + Cpd. 91488, 50 mg/kg	(2)	37	37	27
" " " 100 mg/kg	(3)	43 ± 6	30 ± 5	27 ± 1
" " " 250 mg/kg	(2)	66	20	14
" " " 500 mg/kg	(1)	80	10	10
" " " 1000 mg/kg	(1)	86	7	7
Cpd. 91488, 250 mg/kg	(1)	53	26	21

Mice were given 1.5 μCi/20 g β-^{3}H-histamine i.v.

[a] As determined by isotope dilution.

[b] As determined by thin layer chromatography.

[c] Isotope dilution assays indicated that most (72%) of the label in the acid metabolite fraction was methylimidazole acetic acid; the remainder was imidazole acetic acid and the ribosyl conjugate of imidazole acetic acid.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HLB 00621-04 CM

PERIOD COVERED
October 1, 1978 through September 30, 1979

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
	Theresa Lo	Research Chemist	CM	NHLBI
OTHERS:	Michael A. Beaven	Pharmacologist	CM	NHLBI

COOPERATING UNITS (if any)

Dr. Bayer was a PHS National Research Fellow, Division of Research Grants, NIH, until June 1, 1979

LAB/BRANCH
Cellular Metabolism

SECTION
Cellular Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 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)

Indomethacin and other anti-inflammatory drugs inhibited growth of rat hepatoma cells (HTC) and a nontransformed human diploid fibroblasts in the G_1 phase of the cell cycle. Synchronous growth of both lines occurred after removal of drug as indicated by the sequence of changes in [^{3}H]-thymidine incorporation into DNA, cellular DNA content, mitotic index, and cell number. All (98%) of the cells resumed DNA synthesis following the removal of indomethacin. Further studies revealed that metabolic processes of the cell were altered in a selective manner. The active transport (system A) but not the facilitated transport of amino acids (system L) were suppressed in the presence of indomethacin. Other transport systems investigated, those of the nucleotides and deoxyglucose were unaffected. The effects on transport were apparent within 1 hr and disappeared rapidly once drug was removed. ATP levels, which declined during the growth of control cultures, fell little in cultures arrested with indomethacin.

PHS-6040
(Rev. 10-76)

Project No. Z01 HLB 00621-04 CM

Project Description:

Objectives: We have shown that indomethacin and aspirin in pharmacological doses inhibit growth of a transplantable mast cell tumor (P815) and Lewis lung carcinoma in mouse (Eur. J. Pharmacol. 37: 367, 1976) and that a variety of nonsteroidal anti-inflammatory drugs inhibited growth of transformed (rat hepatoma, HTC) and nontransformed (human fibroblast) cells in culture (J. Pharmacol. Exp. Ther. 202: 446, 1977). Their order of potency in inhibiting growth paralleled their anti-inflammatory activity. Pharmacologically inactive metabolites and derivatives of the anti-inflammatory drugs had little effect on culture growth.

Last year we reported that cultures were arrested in the G_1 phase of the cell cycle and that after removal of drug there was a short delay (18-24 hr) before growth resumed. The time course and sequence of changes in DNA synthesis and cell number indicated that the cultures resumed growth in synchrony.

This year we have begun to study in detail the effect of these drugs on biochemical events and processes that might have some bearing on the possible mechanism of action of these drugs on culture growth. The factors influencing the uptake and distribution of ^{14}C-labeled indomethacin in the cell cultures were also studied.

Methods Employed: Incubation Procedures. HTC cells were grown in Eagle's medium supplemented with Earle's salts, 2 mM glutamine and 10% fetal calf serum in an atmosphere of 95% air and 5% CO_2 at 37°C. For individual experiments, cells from confluent cultures were suspended by shaking the flasks and diluted 20-fold with fresh medium. Samples (1 ml, 50,000 cells) of the diluted suspension were dispensed into individual wells of tissue culture cluster plates (16 mm wells). For the measurement of mitotic index, DNA content, and autoradiographic studies, cultures (10 ml) were grown in petri dishes on glass slides.

Addition of Drugs. Samples, 100 µl or less, of a neutralized solution of the drug (in Eagle's medium) or 10 µl of a solution of indomethacin-^{14}C/1 µCi were added to each well at the times indicated. To remove drug, the medium was aspirated, the cultures were washed once with medium and 1 ml of fresh medium was added. All solutions were sterilized by filtration.

Measurement of Cell Counts, Cell Viability and Mitotic Index. To count cells, the cultures were washed with Ca- and Mg-free Dulbecco's medium and then incubated with a 0.025% w/v trypsin solution. Cells were counted in a Particle Data Counter or a Neubauer counting chamber. Cell viability was assessed by ability to exclude trypan blue (0.05% solution). For mitotic index, the glass slides were fixed with an ethanol-acetic acid mixture (1:3), air-dried, and then treated with Geimsa stain.

Measurement of DNA synthesis. DNA synthesis was measured by the addition of 1 µCi deoxyribose[5-^{3}H]thymidine (5 Ci/mmole, 1 Ci/ml) (Amersham/

Searle Corp., Arlington Heights, Ill.) and incubation of the cultures for 30 min. The amount of isotope incorporated into the trichloracetic acid-precipitable fraction was measured as described in last year's report.

Cytofluorometric and Autoradiographic Studies. The slides were fixed and stained with mithramycin by a modification of the procedure of E. Johannison and B. Thorell (J. Histochem. Cytochem. 25: 122-128, 1977). For autoradiographic studies, the cultures were incubated with tritium-labeled thymidine (60 Ci/mmole), 0.5 μCi/ml, for the indicated times. The slides were washed, fixed, treated with a liquid film emulsion and stored for 4 weeks before development.

Measurement of ^{14}C-Indomethacin Uptake. Culture medium was removed by aspiration and the cells were washed briefly in 0.1 ml ice-cold Dulbecco's medium and then 0.1 ml 0.4 M perchloric acid. The cell residues were washed with 0.1 ml of an ethanolic solution of unlabeled indomethacin (1 mg/ml) to extract labeled indomethacin from the precipitated cell residues.

Measurement of Amino Acid, Glucose and Nucleotide Transport. Experiments were performed in a sterile hood heated to 37°C. Cultures were washed twice in Dulbecco's medium and solutions of α-aminoisobutyric acid [^{3}H], amino-bicycloheptyl-carboxylic acid [^{14}C], deoxyglucose [^{14}C], uridine [^{14}C], thymidine-[^{3}H] or mixtures of these (20 nCi of ^{14}C- and 100 nCi of ^{3}H-labeled compounds) in Dulbecco's medium were added. The cultures were incubated for 10 min unless indicated otherwise, placed on ice, washed twice with ice-cold Dulbecco's medium (Ca^{++} + Mg^{++}-free) and lysed with 200 μl water. Samples (100 μl) of the lysate were assayed for radioactivity. In some experiments, unlabeled amino acids were added along with the labeled compounds.

Assay of Adenylate Nucleotides. These (i.e. ATP, ADP, and AMP) were assayed by use of the Dupont 760 Luminescence Biometer and firefly luciferin-luciferase reaction kit. ADP and AMP were converted to ATP by incubation of samples with appropriate converting enzyme and coreactants prior to addition of the luciferin-luciferase system.

Major Findings: Time Course of Onset and Reversal of Inhibition by Indomethacin. In both exponentially growing HTC and fibroblast cultures 24 hr lapsed before cell proliferation was fully arrested. Upon removal of the drug, there was after a delay of about 24 hr a resumption of exponential growth at a rate similar to that observed in control cultures, with a doubling time of 22 hr for both HTC and fibroblast cultures. Two days after the addition of indomethacin, most of the cells acquired a DNA content characteristic of the G_1 phase of the cell cycle. Upon resupmtion of growth, the distribution of cells in the S and G_2 + M phases of the cell cycle was similar to that of exponentially growing control cultures.

DNA synthesis in indomethacin-inhibited cultures was low and remained depressed for 10 hr after removal of the drug. Thereafter, the rate of synthesis increased rapidly and reached a maximum at 20 hr. A second, slightly broader, peak occurred at 40 hr in the fibroblast culture. A peak increase

in mitotic index was observed by 24 hr in the HTC cultures and 32 hr in the fibroblast cultures. The number of mitotic cells increased sharply and at least 60% of the cells were collected in mitosis. Autoradiographs of HTC cells incubated [^{3}H]-thymidine indicated that by 20 hr almost all (98%) of the cell nuclei were labeled.

Influence of Anti-inflammatory Drugs on Cellular Transport Mechanisms. Of the transport systems studied (nucleotide, glucose and amino acids), only the Na^+-dependent active transport or "A" system of amino acids was inhibited. Transport of uridine, thymidine, deoxyglucose and aminobicycloheptyl carboxylic acid (BCH) (a substrate for the "L", Na^+-independent system) were unaffected. The rate of transport of aminoisobutyric acid (AIB) (a substrate for the "A" system) decayed to low levels 4 hr after the addition of aspirin (5 mM), phenylbutazone (1 mM), sodium meclofenamate (0.5 mM) and indomethacin (0.4 mM). The transport of AIB returned to normal (0.4) 6-7 hr upon removal of drugs.

Kinetic studies indicated that in the presence of indomethacin the rate of influx of AIB was decreased, the value for "K_m" was increased but that V_{max} was unaltered. Our present interpretation of these data is that the mechanism of "energizing" the carrier system was impaired but that the number of "carriers" remained unaltered. However, determination of the number of binding sites for AIB by Scatchard analysis and studies with inhibitors remain to be undertaken.

Changes in Adenylate Nucleotide Levels. In exponentially growing control cultures by 4 days the intracellular level of ATP decreased by 43% (1284 $\pm$ 23 pg/cell on day two vs. 556 $\pm$ 9 pg/cell on day six). In the presence of indomethacin, the rate of ATP depletion was slower and when cell proliferation was completely arrested, the intracellular ATP level of HTC remained relatively constant. Changes in the ratio of ATP/ADP were apparent shortly after the addition of indomethacin but additional studies with uncouplers of oxidative phosphorylation (dinitrophenol) are required to assess the significance of these changes. Incubation of HTC cultures with Antimycin-A and deoxyglucose produced a 90% drop in ATP levels within 10 min.

Studies of ^{14}C-Indomethacin Distribution and Metabolism in HTC Cultures. The drug was taken up and accumulated in the cells within minutes. In the absence of serum, drug levels in the cells were 6-13 times that in the medium. This ratio decreased markedly if the amount of serum in the medium or pH of the medium was increased. The relationship between "uptake" and drug concentration suggested that at least two pools of drug existed in the cell--one saturated at therapeutic concentrations of drug, the other nonsaturable over a wide range of dry concentrations. Accumulation of the drug was reversible. The rate of efflux of drug from cells after transfer to fresh medium was rapid ($t_{1/2}$ < 30 seconds). The studies indicated that retention of drug by the cells was not a factor in the delay in the resumption of culture growth and that serum content and pH of the medium are important factors influencing the cellular content of drug. There were indications that the drug was localized in membrane and lipid structures.

Significance to Biomedical Research: The work reveals a valuable property of indomethacin; that is, it can arrest cell growth in the G_1 phase for up to several days and that after removal of drug synchronous growth of culture occurs. The ability to inhibit proliferation without being cytotoxic may have therapeutic significance, although this remains to be proven, and may have potential use in studies of cell cycle.

Proposed Course: Studies of the effect of indomethacin and other drugs on amino acid transport and ATP production will continue. Reactions associated with changes in membrane function, e.g. methylation of phospholipids, sodium transport, will be investigated as possible mechanisms for the decay in active transport.

Publications:

Bayer, B.M. and Beaven, M.A.: Evidence that indomethacin reversibly inhibits growth in the G_1 phase of the cell cycle. Biochem. Pharmacol. 28: 183-188, 1979.

Bayer, B.M., Kruth, H.S., Vaughan, M. and Beaven, M.A.: Arrest of cultured cells in the G_1 phase of the cell cycle by indomethacin. J. Pharmacol. Exp. Ther., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HLB 00622-02 CM

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Regulation of Cyclic Nucleotide Metabolism

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

PI:	Joel Moss	Staff Investigator	CM	NHLBI
	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI
OTHER:	Peter H. Fishman	Biochemist	LN	NINCDS

COOPERATING UNITS (if any)
Dr. C.R. Alving, Walter Reed Army Inst. of Res., Washinton, D.C.; Drs. S.H. Richardson and S. Garrison, Wake Forest Univ., Winston-Salem, N.C.; Dr. N.J. Oppenheimer, Univ. of California, San Francisco. Dr. P.H. Fishman is with the Lab. of Neurochemistry, NINCDS, NIH.

LAB/BRANCH
Cellular Metabolism

SECTION
Cellular Regulatory Mechanisms

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.6	0.9	1.7

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

1) Choleragen, the toxin that activates adenylate cyclase in vertebrate cells, bound to liposomes containing ganglioside G_{M1} resulting in a perturbation of membrane structure with release of intraliposomal low molecular weight compounds. Binding of toxin to surface G_{M1} effectively protected the ganglioside from labeling by galactose oxidase and NaB^3H_4 or by $NaIO_4$ and NaB^3H_4. Interaction of choleragen with G_{M1} incorporated into liposomes had the same characteristics and specificity noted with biological membranes. 2) Choleragen, a multivalent oligomeric protein, acted as a bridge between G_{M1} molecules on different cells and liposomes, causing their agglutination. Agglutination was not observed with cells that had been treated with G_{M2}, G_{D1a}, or G_{D1b}. The oligosaccharide of G_{M1} prevented agglutination. Choleragen thus exhibited lectin-like properties. 3) Escherichia coli heat-labile enterotoxin increased cAMP in ganglioside-deficient transformed fibroblasts only after they were treated with G_{M1}. It would appear that G_{M1} acted as a receptor for the E. coli toxin. Activation of adenylate cyclase required NAD and involved the stereospecific ADP-ribosylation of cellular protein. The E. coli toxin thus is catalytically equivalent to choleragen.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To define the factors that regulate cyclic nucleotide metabolism in mammalian cells. Cyclic nucleotide synthesis and degradation are regulated in part by mammalian hormones and their analogs and bacterial toxins. Activation of adenylate cyclase by these agents involves their interaction with a surface receptor and subsequently, in the case of two toxins, the enzymatic modification of a cellular protein. The mechanisms involved in the binding of the agents to their receptors and their activation of adenylate cyclase were studied in model membranes, purified systems and cultured cells.

Methods Employed: Liposomal model membranes were prepared by methods published previously. NAD glycohydrolase and ADP-ribosyltransferase activities were determined by assays developed in the laboratory. Tissue culture procedures have been reported previously. Labeling of choleragen, purification of E. coli heat-labile enterotoxin, assays of adenylate cyclase and cAMP, and galactose oxidase-NaB[$^3H_4$] labeling of gangliosides were performed by published procedures.

Major Findings: (1) Interaction of choleragen with membrane receptors incorporated into model systems: The monosialoganglioside G_{M1} has been implicated as the membrane receptor for both choleragen, the bacterial enterotoxin believed to be responsible for the diarrheal syndrome characteristic of cholera. The interaction of choleragen and G_{M1} has been studied in the artificial liposomal model membrane system. Binding of choleragen to liposomal model membranes containing G_{M1} resulted in disruption of the membrane structure and release of trapped low molecular weight compounds. This membrane damage occurred in the presence of the B protomer of the toxin, with no apparent requirement for the A subunit. Binding of ^{125}I-choleragen to liposomal model membranes containing G_{M1} was rapid, not readily reversible and saturable. Half-saturation occurred at 10^{-10} M choleragen and similar concentrations of unlabeled toxin blocked the binding of ^{125}I-choleragen to the liposomes. Binding was highly specific for liposomes containing G_{M1}; only small amounts of toxin bound to liposomes containing G_{M2}, G_{A1}, or G_{D1b}. Choleragen effectively protected the G_{M1} in liposomes from external labeling by sequential treatment with galactose oxidase and NaB$^3H_4$; incorporation of ^{3}H into the terminal galactose moiety of G_{M1} was reduced by 90%; liposomal G_{D1b}, G_{D1ab} and G_{M2} were protected to a lesser extent. Binding of choleragen also reduced labeling of the sialic acid residue of liposomal G_{M1} by $NaIO_4$ and NaB$^3H_4$. These results are similar to those reported for intact cells [Moss et al. (1977) Biochemistry 16, 1976]. Interaction of choleragen with G_{M1} incorporated into lipid model membranes had the same characteristics and specificity noted with biological membranes.

(2) Lectin-like behavior of choleragen: Choleragen is composed of one A subunit and five B subunits; the five B subunits are responsible for binding of the toxin to the oligosaccharide moiety of G_{M1}. The toxin is believed to be capable of multivalent binding, as shown by equilibrium dialysis and gel permeation studies with [^{3}H]G_{M1}-oligosaccharide, and as suggested by its

ability to cause patching and capping on a lymphocyte surface. In addition to cross-linking G_{M1} on the surface of a single cell, choleragen can act as a bridge between G_{M1} molecules on different cells, in effect exhibiting lectin-like behavior. Choleragen agglutinated erythrocytes that had incorporated exogenous G_{M1}; agglutination was not observed when the cells had been exposed to G_{M2}, G_{M3}, G_{D1a} and G_{D1b}. Choleragen also caused the agglutination of liposomes containing ganglioside; liposomes containing either G_{M1} or G_{D1b}, but not G_{M2}, G_{D1a} or G_{M3}, were agglutinated. The oligosaccharide isolated from G_{M1}inhibited both the agglutination of cells and liposomes containing G_{M1} and the binding of choleragen to liposomes containing G_{M1}. Galactose and sialic acid were less effective inhibitors of liposomal agglutination and did not inhibit cellular agglutination or binding of choleragen to liposomes. Liposomal agglutination was dependent on toxin concentration and occurred with the purified B but not the A protomer of choleragen. Choleragen thus exhibits lectin-like behavior which apparently results from a specific interaction of its multiple B subunits with the oligosaccharide moieties of several G_{M1} molecules.

(3) Activation of adenylate cyclase by Escherichia coli heat-labile enterotoxin.

A. Identification of monosialoganglioside G_{M1} as a functional cellular receptor for Escherichia coli heat-labile enterotoxins: Escherichia coli heat-labile enterotoxin (LT) is believed to be involved in the pathogenesis of "traveler's diarrhea." LT cross-reacts immunologically with antisera directed against primarily the B subunits of choleragen; it is through the B subunit that choleragen binds to its specific cell surface receptor, ganglioside G_{M1}. A chemically transformed line of mouse fibroblasts (NCTC 2071, American Type Culture Collection) which is deficient in gangliosides and lacks G_{M1} responded to choleragen with an increase in intracellular cyclic AMP only after the cells had incorporated exogenous G_{M1}. These fibroblasts did not raise their cAMP content in response to E. coli heat-labile enterotoxin. Following the uptake of monosialoganglioside G_{M1}, the cells responded to LT with an increase in intracellular cAMP. The di- and trisialogangliosides which are metabolized to G_{M1} by these cells were considerably less effective. G_{M1} had previously been implicated as the receptor for LT based on the ability of free ganglioside to inhibit the effects of toxin. These investigations establish that the ganglioside, when incorporated into fibroblasts, serves a functional role in mediating responsiveness to toxin.

B. Mechanism of activation of adenylate cyclase by E. coli heat-labile enterotoxin: The effects of LT on cells appear to be secondary to activation of adenylate cyclase and the resulting increase in intracellular cAMP. Activation of adenylate cyclase in cell-free systems by LT requires NAD and thus appears to be similar to cyclase activation by choleragen. In addition, both LT and choleragen catalyze the hydrolysis of NAD to ADP-ribose and nicotinamide and the NAD-dependent ADP-ribosylation of arginine. Analysis of the product of the ADP-ribosylation of arginine by nuclear magnetic resonance spectroscopy indicated that the reaction was stereospecific and resulted in the formation of α-ADP-ribose-L-arginine. This reaction product rapidly

anomerized to yield a mixture of the α and B forms. In the presence of [adenine-U-^{14}C]NAD, LT catalyzed the transfer of radiolabel to proteins; the ADP-ribosylation of proteins was inhibited by arginine methyl ester, an alternative substrate. Digestion of the ^{14}C-protein with snake venom phosphodiesterase released predominantly 5'-AMP. No product was obtained with a mobility similar to that of 2'-(5"-phosphoribosyl)-5'-AMP. This result is consistent with the covalent attachment by the enterotoxin of ADP-ribose, rather than poly(ADP-ribose), to protein. Thus, LT appears to be catalytically equivalent to choleragen and exerts its effects on cells by the stereospecific NAD-dependent ADP-ribosylation of a cellular protein.

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 understend the controls which operate in the more complex pulmonary and cardiovascular system.

Proposed Course: Emphasis will be placed on defining the events which occur following the binding of hormone or toxin to the surface receptor and which lead to activation of adenylate cyclase.

Publications:

Ross, P.S., Moss, J. and Vaughan, M.: Interaction of choleragen and thyrotropin with bovine thyroid adenylate cyclase. Endocrinology 104: 1036-1040, 1979.

Fishman, P.H., Moss, J., Richards, R.L., Brady, R.O., and Alving, C.R.: Liposomes as model membranes for ligand-receptor interactions: Studies with choleragen and glycolipids. Biochemistry, in press.

Richards, R.L., Moss, J., Alving, C.R., Fishman, P.H., and Brady, R.O.: Choleragen (cholera toxin): A bacterial lectin. Proc. Natl. Acad. Sci. USA, in press.

Moss, J., Garrison, S., Oppenheimer, N.J., and Richardson, S.H.: NAD-dependent ADP-ribosylation of arginine and proteins by E. coli heat-labile enterotoxin. J. Biol. Chem., in press.

Moss, J., Garrison, S., Fishman, P.H., and Richardson, S.H.: Gangliosides sensitize unresponsive fibroblasts to E. coli heat-labile enterotoxin. J. Clin. Invest., in press.

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Moss, J. and Richardson, S.H.: Activation of adenylate cyclase by heat-labile Escherichia coli enterotoxin. J. Clin. Invest. 62: 281-285, 1978.

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Vaughan, M. and Moss, J.: ADP-ribosylation and activation of adenylate cyclase. Proceedings of the Fourth US-USSR Symposium on Myocardial Metabolism, September 14-16, 1979, Tashkent, USSR, in press.

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Moss, J., Stanley, S.J. and Oppenheimer, N.J.: Substrate specificity and partial purification of a stereospecific NAD- and guanidine-dependent ADP-ribosyltransferase from avian erythrocytes. J. Biol. Chem., in press.

Moss, J., Fishman, P.H. and Watkins, P.: In vivo degradation of ^{125}I-choleragen by normal human fibroblasts. Proceedings

of the Joint Cholera Conference of the US-Japan Cooperative Medical Science Program, July 23-25, 1979, Bethesda, Md., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HLB 00623-01 CM

PERIOD COVERED

October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Mevalonate Kinin and Phosphomevalonate Kinase in Human Fibroblasts and Rat Liver

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

PI:	Earl D. Mitchell, Jr.	Research Chemist	CM	NHLBI
	Joel Avigan	Research Chemist	CM	NHLBI

COOPERATING UNITS (if any)

Dr. Mitchell was assigned from Oklahoma State University under the Intergovernmental Personnel Act of 1970.

LAB/BRANCH

Cellular Metabolism

SECTION

Cellular Regulatory Mechanisms

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	0.8	0.4

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Regulation of mevalonate kinase activity was studied in human skin fibroblasts grown in tissue culture and in rat liver. Phosphomevalonate kinase, another enzyme in the biosynthetic pathway of cholesterol, was also assayed in rat liver. The activity of both enzymes was reduced by over 50% in livers of cholesterol-fed rats. In fibroblasts, there was twice as much mevalonate kinase activity following incubation with medium containing solvent-extracted serum as in cells treated with whole serum. There was no such reduction in activity due to serum lipids in fibroblasts derived from a patient with homozygous familial hypercholesterolemia. The activity of mevalonate kinase was increased following incubation with insulin. It appears that the above two enzymes could play a role in the physiological regulation of sterol synthesis.

PHS-6040
(Rev. 10-76)

Project No. Z01 HLB 00623-01 CM

Project Description:

Objectives: The purpose of this study is to explore the potential role of certain enzymatic steps, other than the reduction of hydroxymethylglutarylcoenzyme A, in the regulation of sterol synthesis in mammalian tissues.

Methods Employed: The methods included techniques previously developed in this laboratory for studies utilizing cells in tissue culture, as well as adaptations of assays for mevalonate kinase and phopphomevalonate kinase that had been introduced by Dr. E. D. Mitchell in his earlier work. They required growing and treatment of skin fibroblasts followed by their washing, harvesting and freezing. The cells were homogenized and the 10,000 x g supernatant was used in incubation with labeled mevalonic acid. Phosphorylated products were separated on small columns containing anion exchange resin and aliquots of effluents were counted. In rat liver, mevalonate kinase was assayed in the 100,000 x g supernatant fraction of the homogenate. Labeled phosphomevalonic acid was used as a substrate in the assay of phosphomevalonate kinase.

Major Findings: The only labeled products found following the assays described above were mevalonic phosphate and pyrophosphate and isopentenyl pyrophosphate. Since the sum of the amounts of these compounds yielded a good recovery of the substrate utilized, it could serve as a measure of the enzyme activity.

Fibroblasts incubated with medium containing 10% fetal calf serum had approximately 50% of the mevalonate kinase activity of cells treated with 10% solvent-extracted serum. For each type of medium, sparse cultures had more activity relative to cell protein than confluent ones. Cells derived from a patient with homozygous familial hypercholesterolemia did not have lower kinase activity when treated with whole serum than when incubated with solvent-extracted serum. Suspensions of cholesterol and of 25-OH cholesterol, however, depressed activities in both normal and familial hypercholesterolemia cell cultures that had been previously stimulated by exposure to a medium with solvent-extracted serum. Insulin increased mevalonate kinase in fibroblasts by as much as 100%. Rat livers from cholesterol-fed animals (1% for 10 days) had less than half the activity of both kinases of livers of control rats.

Significance to Biomedical Research: It has been generally recognized that cholesterol synthesis in mammalian organisms is readily affected by changes in the activity of hydroxymethylglutaryl coenzyme A reductase. It is not established whether changes in activities of other enzymes on the biosynthetic pathway of cholesterol contribute to the physiological regulation of this process. The current study suggests such a possibility.

Proposed Course: A study of the dependency of the stimulation of mevalonate kinase activity by delipidated serum on protein synthesis and of the enzyme turnover rate is currently being conducted. It is expected that the project will be terminated with the completion of these experiments.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HLB 00624-01 CM

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
The Effect of Steroids on Cholesterol Synthesis and Esterification in Human Skin Fibroblasts in Tissue 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)

None

LAB/BRANCH
Cellular Metabolism

SECTION
Cellular Regulatory Mechanisms

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	0.5	0.6

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The effects of female sex hormones and glucocorticoids on the synthesis of free and esterified cholesterol in human skin fibroblasts is being investigated. The results show a significant influence of these steroids on various aspects of cholesterol metabolism.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives and backgrounds: Sex hormones and glucocorticoids affect cholesterol mobilization and biosynthesis in the respective target organs in vivo. The biochemical mechanisms of these effects are far from clear. Although skin fibroblasts may not be specific target cells of the steroids of interest, it is quite possible that under in vitro conditions, in which their uptake may be induced by maintaining pharmacological concentrations of the compounds under study, the biochemical effects would be analogous to these observed in specific organs and cells that cannot be conveniently maintained in culture.

Methods Employed: Human skin fibroblasts are grown in monolayers by routine methods and incubated in media with or without the steroids under study. Following these preparatory steps, the various assays are carried out to measure the enzymatic activities of interest.

Major Findings: In agreement with published reports, progesterone, a 20 ug/ml, present for one or two days diminished esterified cholesterol content and inhibited the rate of cholesterol esterification, as measured with labeled oleic acid. However, in experiments in which the steroid was allowed to act for 4 days, the rate of cholesterol esterification was greatly increased (10 to 11-fold). These variable effects may reflect an interplay of several factors, including that of a growing pool of unesterified cholesterol which is known to trigger esterification--a process that is delayed in the presence of progesterone. When cells were loaded with esterified cholesterol by incubation with LDL, and subsequently incubated in the presence of HDL-3 as a sterol acceptor, progesterone enhanced the decline in total cellular sterol, possibly because unesterified cholesterol, maintained in these cells at higher levels, is more readily transported into the medium than its esterified form, which was accumulated in the absence of progesterone. Dexamethasone did not affect significantly the cellular content of esterified cholesterol, but like progesterone it increased the rate of cholesterol esterification following a 4-day incubation. Somewhat similar effects were observed with ethynyl estradiol.

The effects of the above steroids on the rate of nonsaponifiable lipid synthesis, as measured with labeled acetate, were greatly dependent on their concentration. In stationary fibroblasts cultures, progesterone did not affect the process within 24 hr at concentration of 0.5 ug/ml, but at 2.5 ug/ml it almost doubled it and stimulated it 12-fold at 20 ug/ml. Most of the increased radioactivity in the nonsaponifiable lipids was associated with chromatographically isolated cholesterol. A similar stimulation was observed in growing, nonconfluent cultures. Dexamethasone, at relatively low concentration of 0.4-1.0 ug/ml, tended to reduce the synthesis of nonsaponifiable lipids by 20-25% (in apparent conflict with results that were previously reported by us based on experiments done with a different cell line). At higher concentrations and in agreement with the previous results, the steroid produced an increase in synthetic activity, reaching a stimulation of 390% at 50 ug/ml. The variable effects of dexamethasone are reflected

in seemingly confusing data found in literature, and they may possibly depend on an interplay of two or more factors influenced by it. In particular, a significant relationship may exist between the effect on cellular growth and the observed stimulation of sterol synthesis.

Significance to Biomedical Research: The clarification of any aspect of the impact of hormones on cholesterol transport and synthesis is potentially valuable for the understanding of important biomedical problems.

Proposed Course: More experimental work remains to be done in order to derive unifying and generalized conclusions from the observations that have been made so far. In particular, the relationship between the effect on cholesterol esterification rate and the effect on cholesterol synthesis should be determined. Also, the relationship between cellular proliferation and sterol synthesis could be advantageously studied applying the results obtained with progesterone and with glucocorticoids. The actual amounts of these steroids taken up by the fibroblasts under the experimental conditions should also be determined.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HLB 00625-01 CM

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
ADP-Ribosyltransferases: Characterization of Their Substrates and of 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	Staff Investigator	CM	NHLBI
	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Cellular Metabolism

SECTION
Cellular Regulatory Mechanisms

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

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1.2	1.2	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Choleragen catalyzes the transfer of ADP-ribose from NAD to multiple soluble and membrane proteins of bovine thymus and brain, as well as to purified proteins such as lysozyme and histone. The product of this reaction is mono-ADP-ribosyl protein. A transferase isolated from turkey erythrocytes catalyzes a similar reaction.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Cholera enterotoxin (choleragen) and a transferase isolated from turkey erythrocytes catalyze the NAD-dependent ADP-ribosylation of proteins. The purpose of this study is to examine the effect of ADP-ribosylation of cellular proteins on metabolic processes and mechanisms for the regulation of toxin- or transferase-catalyzed ADP-ribosylation of endogenous proteins.

Methods Employed: Purified proteins, or fractions from bovine thymus or brain are incubated with ^{32}P- or ^{3}H-NAD; ADP-ribosylated proteins are precipitated with acid and collected on filters. Mixtures of ADP-ribosylated proteins are separated on polyacrylamide slab gels containing SDS and labeled proteins identified by radioautography. ADP-ribosylated proteins are digested with snake venom phosphodiesterase to study the nature of the covalent modification.

Major Findings: Choleragen catalyzes the transfer of the ADP-ribose moiety of NAD to purified proteins, e.g., lysozyme and histones, as well as endogenous proteins of bovine thymus and brain. Several soluble and membrane proteins are labeled with ADP-ribose in both tissues, as evidenced by multiple bands on radioautographs of 12% slab gels containing SDS. This is in contrast to the pigeon erythrocyte system in which a single protein is the predominantly labeled species. The ADP-ribosylation of several brain and thymus proteins is observed with turkey erythrocyte transferase as well as with cholera toxin. Using a preparation of soluble proteins from thymus as substrate, the apparent Km for NAD is 3 mM for choleragen and $\sim$ 1 mM for turkey transferase. Analysis of proteins ADP-ribosylated by either enzyme via digestion with snake venom phosphodiesterase revealed that the product is a single ADP-ribose unit covalently bound to the acceptor protein. This is in contrast to the product of the endogenous nuclear enzyme poly-ADP-ribose polymerase, in which a polymer of ADP-ribose is covalently attached to protein.

Significance to Biomedical Research: Choleragen exerts its effects on mammalian cells by activating adenylate cyclase, thereby increasing intracellular cAMP levels. This presumably occurs via the toxin-catalyzed ADP-ribosylation of a component of the adenylate cyclase system. The elucidation of the nature of the specific cyclase component modified by choleragen will greatly extend present knowledge of the regulation of cyclic nucleotide metabolism. Moreover, the presence of an endogenous enzyme in turkey erythrocytes that catalyzes a reaction identical to that catalyzed by cholera toxin suggests that a similar mode of regulation may exist endogenously. Since cyclic nucleotides are known to regulate numerous metabolic processes in tissue such as heart and lung, knowledge of the regulation of cAMP levels would be valuable.

Proposed Course: 1) Identification of the specific ADP-ribosylated protein involved in the adenylate cyclase system.

2) Study of factors that modulate the cholera toxin- or transferase-catalyzed reaction.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HLB 00626-01 CM

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Role of Guanine Nucleotides in Regulation of Adenylate Cyclase Activity

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

PI:	Seishi Nakaya	Visiting Associate	CM	NHLBI
	Joel Moss	Staff Investigator	CM	NHLBI
	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Cellular Metabolism

SECTION
Cellular Regulatory Mechanisms

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)

GTP is required for the NAD-dependent activation of adenylate cyclase by choleragen for stabilization of the activated enzyme and for its optional catalytic function in cAMP synthesis. GTP is more potent than ITP or ATP, and its effects are enhanced in each case by the presence of a nucleotide regenerating system.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To define the role of guanine nucleotides in regulation of adenylate cyclase activity.

Methods Employed: Adenylate cyclase and protein were assayed by established procedures. The bovine brain particulate fraction was purified by methods developed in the laboratory.

Major Findings: In cell-free preparations, activation of adenylate cyclase by choleragen is dependent on NAD and GTP. NAD serves as an ADP-ribose do not in a toxin-catalyzed ADP-ribosylation reaction, as shown in this laboratory. The ADP-ribose acceptor appears to be, in several systems, a 42,000 dalton protein which possesses a GTP-binding site. Since GTP is critical to the expression of both hormone- and toxin-stimulated adenylate cyclase activity, and since resolution of the 42,000 dalton protein from the cyclase catalytic unit results in a loss of GTP responsiveness, it has been proposed that choleragen causes activation of adenylate by the NAD-dependent ADP-ribosylation of the GTP-binding subunit of cyclase.

Choleragen activates bovine brain adenylate cyclase in a reaction dependent on NAD and GTP. To investigate further the role of GTP in the adenylate cyclase system, the activation process and the assay of cyclase catalytic activity were carried out separately in two incubations. First, the particulate fraction was activated by choleragen and NAD (Incubation I). The particulate fraction was then washed and assayed with either [^{32}P] App(NH)p or [^{32}P]ATP as substrate (Incubation II); Incubation II was independent of toxin or NAD. Both the activation and assay required nucleoside triphosphate with GTP > ITP > ATP. The apparent K_a for GTP in either Incubation I or II was decreased by a nucleoside triphosphate regenerating system, such as pyruvate kinase and phosphoenolpyruvate. The choleragen-activated cyclase, following the first incubation, was stable at 0° but unstable at 30°. GTP stabilized the cyclase at 30° and was more effective than ITP or ATP. The bovine brain adenylate cyclase thus requires GTP for activation by choleragen, stabilization of the activated enzyme, and expression of the catalytic activity in the assay.

Significance to Biomedical Research: Many hormones and toxins exert their effects on cells through the activation of adenylate cyclase in a GTP-dependent process. The resulting increase in intracellular cAMP has diverse metabolic effects dependent on both hormone and tissue. Knowledge as to the mechanism of these effects would have to have more precise pharmacological manipulation.

Proposed Course: To purify, after solubilization, the adenylate cyclase and associated regulatory proteins in order to define the mechanism of action of GTP and other molecules in the control of catalytic activity.

Publications: Nakaya, S., Moss, J. and Vaughan, M.: Analysis of cofactor requirements for activation of adenylate cyclase by cholera-

gen independent of requirements for catalytic activity. In Proceedings of the Fifteenth Joint Cholera Conference of the US-Japan Cooperative Medical Science Program, July 23-25, 1979, Bethesda, Md., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HLB 00627-01 CM

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Role of GTPase in Regulation of Adenylate Cyclase Activity

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
	Joel Moss	Staff Investigator	CM	NHLBI
	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cellular Metabolism

SECTION
Cellular Regulatory Mechanisms

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

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

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

It has been reported that turkey erythrocytes contain a guanosine triphosphatase (GTPase) with a very low K_m for GTP that can be activated by catecholamines. This activated GTPase is inhibited by cholera toxin. To evaluate the hypothesis of Cassel and Selinger that this GTPase is a regulatory moiety of the adenylate cyclase complex, we are examining in parallel the effects of prostaglandins, catecholamines, toxin, and other agents on GTPase and adenylate cyclase activities in human mononuclear cell membranes and turkey erythrocyte membranes.

PHS-6040
(Rev. 10-76)

Project No. Z01 HLB 00627-01 CM

Project Description:

Objectives: It has been reported that turkey erythrocytes contain a guanosine triphosphatase (GTPase) with a very low K_m for GTP that can be activated by catecholamines. This activated GTPase is inhibited by cholera toxin. To evaluate the hypothesis of Cassel and Selinger that this GTPase is a regulatory moiety of the adenylate cyclase complex, we are examining in parallel the effects of prostaglandins, catecholamines, toxin, and other agents on GTPase and adenylate cyclase activities in human mononuclear cell membranes and turkey erythrocyte membranes.

Methods Employed: Human mononuclear cells were prepared from whole blood by centrifugation through Ficoll-Paque gradients. Particulate fractions (membranes) were prepared by homogenization and centrifugation at 25,000 x g. Turkey erythrocyte membranes were prepared by lysing the erythrocytes followed by DNase treatment as described as Schramm and Rodbell (J. Biol. Chem. 250: 2232, 1975). GTPase was assayed by measuring the liberation of ^{32}P; from [γ-^{32}P]GTP as described by Cassell and Selinger (Biochem. Biophys. Res. Commun. 77: 868, 1977). Adenylate cyclase was assayed by the procedure of Salomon and Rodbell (Anal. Biochem. 58: 541, 1974).

Major Findings: Human mononuclear all membranes have a GTPase activity which was stimulated on average of 25 to 30% with PGE_1. Other prostaglandins (PGA_1, PGB_1, PGE_2, $PGF_{1\alpha}$) also stimulated the GTPase. Cholera toxin had no significant effect on GTPase in the presence or absence of PGE_1. The GTPase was not stimulated by isoproterenol. PGE_1 did not stimulate GTPase in turkey erythrocyte membranes.

Mononuclear cell membrane adenylate cyclase was stimulated 5- to 10-fold by PGE_1. Other prostaglandins (PGA_1, PGB_1, PGE_2) also stimulated adenylate cyclase. $PGF_{1\alpha}$ had no effect on the adenylate cyclase. Isoproterenol produced a twofold increase in adenylate cyclase. PGE_1 did not stimulate the adenylate cyclase in turkey erythrocyte membranes.

Significance to Biomedical Research: Many hormones, drugs, and other agents exert their effects on cells by modifying adenylate cyclase activity, but the molecular mechanisms through which this important enzyme is regulated remain to be elucidated.

Proposed Course: To define the relationship of the specific GTPase to adenylate cyclase and its role in the regulation of cyclase activity.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HLB 00628-01 CM

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Calmodulin and Troponin C: Comparison of Activities in Phosphodiesterase and ATPase Systems

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
	Vincent C. Manganiello	Medical Officer (Res.)	CM	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cellular Metabolism

SECTION
Cellular Regulatory Mechanisms

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Regulatory mechanisms of cGMP phosphodiesterases in bovine brain and rat liver are studied. In bovine brain the calcium-binding protein which increases phosphodiesterase activity (calmodulin) can substitute for troponin C in the actomyosin Ca-ATPase system. cGMP phosphodiesterase may be regulated by a complex similar to the troponin complex in skeletal muscle. Rat liver contains a cGMP phosphodiesterase activity which is not increased in the presence of calcium and the calcium-binding protein. This enzyme differs from the activatable cGMP phosphodiesterase in molecular weight and kinetic properties.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Mammalian tissues contain specific cGMP phosphodiesterase, the activities of which are strictly regulated. In bovine brain, phosphodiesterase activity increased in the presence of calcium and a calcium-binding protein (calmodulin) can also be inhibited by other modulators. It is possible that these regulatory elements exist as a complex similar to the troponin-tropomyosin complex found in skeletal muscle. Other workers have noted many similarities between calmodulin and troponin C. Isolation from bovine brain of a troponin-like complex regulating actomyosin Ca-ATPase activity has been reported (Mahendran and Berl, 1977). The objective of this project is to determine whether a similar complex mdoulating the activity of cGMP phosphodiesterase can be found in bovine brain. Subsequent characterization of the individual elements of such a complex can be made with some comparison made with the components of the troponin system.

Methods Employed: Purified components of the muscle troponin-tropomyosin system were kindly provided by Dr. P. Levis. A previously published method for isolating the troponin-like complex from brain was followed (Mahendran and Berl, 1977). Fresh bovine brain was homogenized and the supernatant from centrifugation saved for ammonium sulfate precipitation. The fraction that precipitated between 40 and 60% saturated ammonium sulfate was solubilized in 1 mM phosphate pH 7.0/1 M KCl/2 mM DTT. It was then loaded onto a hydroxylapatite column equilibrated with the same buffer and eluted with a gradient from 1 to 200 mM phosphate/1 M KCl. Fractions were then analyzed as effectors of both the cGMP phosphodiesterase activity and actomyosin Ca-ATPase activity.

Major Findings: Purified troponin components are capable of modulating cGMP phosphodiesterase activity from bovine brain. In the presence of calcium, troponin C can increase the activity of the enzyme, while in the absence of calcium, troponin I and troponin T inhibit phosphodiesterase activity. Calmodulin from bovine brain can substitute for troponin C in forming the troponin-tropomyosin complex and produces calcium-sensitive activation of actomyosin ATPase.

Proposed Course: This project will be terminated when current experiments are completed.

Significance to Biomedical Research: Elucidation of the mechanism of action of Ca^{2+} in modulation of enzyme reactions in vitro is of major importance in understanding the regulation of many cellular processes in which Ca^{2+} plays a key role.

Publications: None

Annual Report of
LABORATORY OF CHEMICAL PHARMACOLOGY
National Heart, Lung, and Blood Institute
October 1, 1978 to September 30, 1979

In recent years this Laboratory has discovered that several drugs and other foreign compounds cause tissue lesions, such as liver and lung necrosis, through the formation of chemically reactive metabolites. Among these compounds are halogenated benzenes, acetaminophen, phenacetin, isoniazid, iproniazid and furosemide. The chemically reactive metabolites, however, have been difficult to identify because of their chemical instability. Indeed, most of our knowledge concerning their identity is based on indirect evidence. During the past year, the major objective of the Laboratory has been to study the mechanisms by which chemically reactive metabolites are formed and eliminated.

Chemically Reactive Metabolites

Pathways of phenacetin and acetaminophen activation. Previous studies have shown that large doses of these commonly used analgesic drugs cause liver necrosis through the formation of chemically reactive metabolites. Last year we reported that phenacetin could be converted to chemically reactive metabolites that react with glutathione through at least three different pathways: 1) Phenacetin undergoes oxidative dealkylation to form acetaminophen, which in turn is converted to a chemically reactive metabolite. 2) Phenacetin is converted to phenacetin-3,4-epoxide which spontaneously loses its ethyl group and reacts with glutathione. 3) Phenacetin is oxidized to N-hydroxyphenacetin and in turn is converted to phenacetin-N-sulfate or phenacetin-N-O-glucuronide which decompose to chemically reactive metabolites. Studies with ^{18}O-labeled phenacetin revealed that the major pathway for the formation of acetaminophen mercapturic acid in hamsters is through the formation of acetaminophen. Although we postulated several years ago that acetaminophen is converted to a chemically reactive metabolite through the formation of N-hydroxyacetaminophen, it is now evident that this is not the mechanism. During the past year, we have developed a method for assaying N-hydroxyacetaminophen in the presence of other N-hydroxyarylacetamides and have shown that N-hydroxyacetaminophen may be formed by oxidative dealkylation of N-hydroxyphenacetin but not from acetaminophen. Moreover, we discovered that liver microsomes from hamsters convert acetaminophen to 3-hydroxyacetaminophen. Since studies with $^{18}O_2$ indicate that ^{18}O is incorporated into the metabolites, it presumably is formed by way of acetaminophen-2,3-epoxide. But this epoxide does not appear to be the major chemically reactive metabolite of acetaminophen, because glutathione, which forms a conjugate with the active metabolite of acetaminophen, does not affect the rate of formation of 3-hydroxyacetaminophen. Moreover, the covalent binding of acetaminophen does not result from the further oxidation of the catechol because neither superoxide dismutase, nor a combination of S-adenosyl methionine and catechol-O-methyl transferase inhibits the covalent binding. Thus, the mechanism of activation of acetaminophen remains obscure.

Dehalogenation reactions. In previous years, we have shown that chloroform is converted by cytochrome P-450 enzymes in rat liver microsomes to a chemically reactive metabolite that either reacts with cysteine to form 2-oxothiazolidine-4-carboxylic acid or decomposes to carbon dioxide. Studies with $^{18}O_2$ revealed that the oxygen in the thiazolidine ring originates from the atmosphere. It, therefore, seemed likely that the chemically reactive metabolite is Cl_3COH which decomposes to phosgene (Cl_2CO). Deuteriated chloroform (Cl_3CD), however, is converted by rat liver microsomes to the chemically reactive metabolite about 50% as rapidly as chloroform. After simultaneous administration of equimolar amounts of $Cl_3{}^{13}CH$ and $Cl_3{}^{12}CD$ to rats and the subsequent isolation of 2-oxothiazolidine carboxylic acid from rat liver, it was found that the ratio of the ^{13}C-labeled to the ^{12}C-labeled 2-oxothiazolidine carboxylic acid was about two, indicating that the isotope effect in the activation of chloroform observed in vitro also occurs in vivo. These findings, together with the observation that Cl_3CD is about half as toxic as Cl_3CH, represents the best evidence thus far obtained that chloroform causes liver necrosis through the formation of phosgene. Other evidence includes the observation that pretreatment of rats with phenobarbital markedly increases both phosgene formation by rat liver microsomes and the liver necrosis caused by chloroform.

As with chloroform, the deuterated forms of bromoform and iodoform are converted to chemically reactive metabolites by a cytochrome P-450 enzyme in rat liver microsomes less rapidly than are their protonated forms. Our finding that Br_3CD and I_3CD are less toxic than Br_3CH and I_3CH thus implies that these compounds cause liver necrosis through the formation of Br_2CO and I_2CO, respectively.

Nevertheless, the deuterated form of halothane (F_3C-BrClD) is about as toxic as its protonated form. Studies during the past year, however, raise the possibility that halothane may be converted to chemically reactive metabolites by liver microsomes by at least two different mechanisms. 1) In air, halothane is converted to a chemically reactive metabolite that no longer contains hydrogen; the radiolabel of ^{14}C-halothane becomes covalently bound to protein and lipid to a much greater extent than the radiolabel of ^{3}H-halothane. 2) In nitrogen, halothane may be converted to several chemically reactive metabolites, at least one of which retains a hydrogen; the radiolabel of ^{3}H-halothane is about one third to one half that of ^{14}C-halothane. Thus, the mechanism of toxicity of halothane may be closer to that of carbon tetrachloride than it is to that of chloroform.

In calves and rats, the dichloroacetyl group of chloramphenicol enters the two-carbon pool and thence is converted in part to serine and glycine. In searching for the mechanism by which this occurs we discovered that a glutathione dependent enzyme in the soluble fraction of rat liver converts chloramphenicol to two metabolites. During the past year, we have proved that one of these metabolites is chloramphenicol aldehyde (R-NH-CO-CHO) and the other is a chloramphenicol oxamyl derivative, probably of glutathione (R-NH-CO-CO-SG). Pretreatment of rats with either phenobarbital or 3-methylcholanthrene does not change the activity of the enzyme.

Toxicity of chloramphenicol and thiamphenicol. A major reason for the un-

popularity of chloramphenicol as an antibiotic is the fact that it causes aplastic anemia. Since thiamphenicol apparently does not cause this kind of toxicity in man, we have compared the toxicity of these drugs in various systems. Both drugs administered orally for 10 days to bovine calves cause aplastic anemia at 6 weeks, but they are much less toxic when administered intravenously. These findings raised the possibility that the aplastic anemia might be due in part to a decrease in the intestinal flora which synthesized vitamins. In accord with this view the aplastic anemia caused by thiamphenicol could be prevented by administration of a vitamin B complex mixture, containing folic acid and vitamin B_{12}. By contrast, the administration of the vitamin B complex decreased but did not entirely prevent the aplastic anemia caused by chloramphenicol. These findings raise the possibility that chloramphenicol causes aplastic anemia in calves not only by interfering with vitamin synthesis but also by another mechanism, perhaps through a chemically reactive metabolite produced by the bacteria in the ruminant stomach.

Toxicity of phenylbutazone. Clinicians have observed that this drug causes liver and kidney damage in about 0.3% of the patients receiving it. During the past two years we have found that large doses (150 mg/kg; i.p.) in rats can cause necrosis in both organs, but probably through different mechanisms. The kidney damage may be caused directly by phenylbutazone or by one of its stable metabolites, oxyphenbutazone. But the liver necrosis apparently is caused through the formation of a chemically reactive metabolite; because both covalent binding of ^{14}C-phenylbutazone and liver necrosis are increased in phenobarbital treated rats, and decreased by injection of piperonyl butoxide. The toxic reactive metabolite apparently is inactivated by glutathione because treatment of the rats with diethyl maleate, which enhances the depletion of hepatic glutathione, increases the toxicity. In accord with these results, diethyl maleate increases the covalent binding in vivo and glutathione decreases the covalent binding in vitro. By contrast, pretreatment of rats with 3-methylcholanthrene increases the formation of another chemically reactive metabolite which does not cause liver necrosis. This chemically reactive metabolite differs from the toxic metabolite in that it does not deplete the liver of glutathione in vivo nor does glutathione prevent its covalent binding in vitro. Both chemically reactive metabolites are formed by cytochrome P-450 enzymes, but neither metabolite has been identified.

Toxicity of paraquat. The mechanism by which this herbicide causes lung toxicity continues to elude us. Most investigators believe that it causes lung toxicity through the formation of hydroxyl radicals formed from superoxide and hydrogen peroxide by the Haber-Weiss reaction. However, at the average concentration of paraquat found in lung of animals given toxic doses of the herbicide, the formation of superoxide by lung microsomes is barely detectable in vitro. Studies during the past year have revealed, however, that paraquat is preferentially transported into lung type II cells, suggesting that the concentration of paraquat within these cells could be high enough to produce significant amounts of superoxide. Nevertheless, the administration of 1-phenyl-3-(2-thiazolyl)-2-thiourea, a hydroxyl radical scavanger, failed to prevent the lung damage caused by paraquat in mice.

Toxicity of diphenylhydantoin. This drug causes gingival hyperplasia in children and ferrets and cleft palate in mice. Studies during the past year indicated that difluorodiphenylhydantoin was at least as effective as diphenylhydantoin in causing cleft palate in fetuses of mice even though metabolism of difluorodiphenylhydantoin could not be detected. Thus formation of cleft palate does not appear to be mediated through the formation of a metabolite of the drug.

Pharmacokinetics

In the past, simple pharmacokinetic models have been invaluable in reconciling seemingly discordant data in our toxicology studies. Although these simple models continue to provide useful ways of comparing data and evaluating the contributions of blood flow rates, reversible binding of drugs to plasma proteins and enzyme activities in various animal tissues, in limiting the metabolism of the parent drugs, a need has arisen for the development of similar models for the rapid formation and elimination of drug metabolites. During the past few years, we have derived the equations for several simple models and are in the process of evaluating their validity.

Theoretical pharmacokinetic relationships. Other workers have pointed out that when a drug is eliminated from the body solely by the liver by a first-order process and is completely absorbed from the gastrointestinal tract, the extraction ratio $[1-(C_{out}/C_{in})]$ of the drug by the liver and the blood flow rate through the liver may be estimated from the area under the concentration-time curve of the drug in systemic blood after intravenous and oral administration of the drug. Other relationships, however, may be estimated from the area under the curve of the primary metabolites as well as the parent drug when the drug and the metabolites are eliminated solely by the liver by first-order reactions and the liver may be viewed as a "well-mixed" pool, in which all hepatocytes contain the same concentration of parent drug and the same concentration of the metabolite. For example, 1) the area under the curve of the metabolite should be independent of the route of administration of the parent drug. Thus, a comparison of the area under the curves of the metabolite may be used to estimate the bioavailability of the drug. 2) The ratio of the AUC of the unbound concentration of the metabolite to that of the parent drug administered orally is independent of the bioavailability of the parent drug but represents the ratio of the actitivites of the enzymes that form and eliminate the metabolite; in other words, the ratio of AUC values will be independent of the other pathways of elimination of the parent drug. By contrast, the ratio of the AUC's after intravenous administration of the parent drug will depend not only on the ratio of the activities of the enzymes that form and eliminate the metabolite, but also on the availability of the metabolite. 3) When both a parent drug, and one of its primary metabolites are administered orally in molar equivalent doses, the ratio of the AUC of the metabolite generated from the parent drug to the AUC of the preformed metabolite should represent the fraction of the dose of the parent compound that is converted in the metabolite. By contrast, when both the parent drug and one of its primary metabolites is administered intravenously, the ratio of the AUC of the generated metabolite to that of the performed metabolite depends not on the fraction of the dose converted

to the metabolite but also on the availability of the generated metabolite. 4) When a parent drug is eliminated from the body by being metabolized in the liver and excreted into the urine, the ratio of the AUC of any one of the primary metabolites of drug after oral and intravenous administration of the drug should equal one plus the clearance of the drug by the kidney divided by the hepatic blood flow (i.e. $[1 + (Cl_R/Q_H)]$, provided that all processes are first-order. Thus, the ratio of the unchanged drug in the urine after oral and intravenous administration of the drug should be the availability (C_{out}/C_{in}) of the parent drug in the liver and the ratio of the metabolites excreted into urine should be $(1 + Cl_R/Q_H)$ when all processes are first-order.

Since many of the relationships described above depend on the validity of the "well-stirred" model, our studies to evaluate the model are being continued with studies of the conversion of acetanilide to acetaminophen. In addition, we have invented ways of calculating the rate constant of elimination of a metabolite when it nearly equals or exceeds the rate constant of elimination of the parent drug. These techniques will prove invaluable in assessing the results of nonsteady-state experiments.

Potentiation of drug toxicity by isoproterenol. Previous studies have revealed that large doses (0.3 mg/kg, s.c.) of isoproterenol potentiate the toxicity of paraquat by slowing its excretion into urine and that this effect can be blocked by β-adrenergic agents, such as propranolol. In addition, isoproterenol decreases the clearance of methoxy inulin, tetraethyl ammonium, p-aminohippurate and furosemide. Indeed, it antagonizes the diuretic effects of furosemide in rats. Since these effects of isoproterenol were not prevented by hypophysectomy, they are probably not mediated by an increase in the release of antidiuretic hormone from the posterior pituitary gland. Although the dose used in rats is several orders of magnitude greater than those used in man, it is noteworthy that other investigators have reported similar changes in human renal function during infusion of isoproterenol in doses as low as 0.04 $\mu g\ kg^{-1}min^{-1}$, which are within the dosage range recommended in the Physicians Desk Reference. It thus appears that humans may be as much as 100 times more sensitive to isoproterenol than rats, based on reported plasma concentrations of isoproterenol.

Effects of glycofurol on drug metabolism. Last year we reported that the administration of glycofurol to rats increased the amount of cytochrome P-450 in liver. During the past year, we have found that a pattern of changes in enzymatic activity could not be accounted for by increases in either the form of cytochrome P-450 induced by phenobarbital (LM-2) or the form induced by 3-methylcholanthrene (LM-4). Instead, electrophoretic evidence suggests that glycofurol induces the synthesis of a unique form of cytochrome P-450. Studies in collaboration with W. Levin of Hoffmann-La Roche with antibodies against LM-2 and LM-4 have confirmed this view.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00806-04 LCP

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Activation of N-Hydroxy-N-Arylacetamides by Sulfation and Glucurondiation

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

PI:	Jack A. Hinson	Staff Fellow	LCP	NHLBI
	James R. Gillette	Chief, Lab. of Chemical Pharm.	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:
0.4	0.4	

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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)

Previously we showed that (^{14}C-acetyl) phenacetin N-O-glucuronide in Tris buffer (t-1/2 = 8.7 hr) decomposed to phenacetin, 2-hydroxyphenacetin glucuronide, acetaminophen, and acetamide. In the presence of protein, covalent binding occurred and only acetaminophen and acetamide were decreased. It was thus postulated that acetaminophen, acetamide and covalent binding were formed from N-acetylimidoquinone. In phosphate buffer the N-O-glucuronide is also converted to 3-hydroxyphenacetin phosphate at the expense of covalent binding, acetaminophen and acetamide. In the absence of protein, phosphate completely inhibits acetamide formation but only partially inhibits acetaminophen formation. In the presence of protein, however, phosphate completely inhibits acetaminophen and acetamide and only partially inhibits covalent binding. These data indicate that three reactive intermediates are formed - an ethylated metabolite which reacts with phosphate and two deethylated metabolites which can bind to protein. Only one reactive intermediate which binds to protein can be inhibited by phosphate. The other reactive metabolite which cannot be inhibited by phosphate does not lead to acetamide but can be reduced to acetaminophen.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Various N-arylacetamides may produce nephrotoxicity, hepatotoxicity or cancer. Reactive metabolites are believed to be important in each of these toxicities. To help elucidate these mechanisms toxicity we have been studying the formation of reactive metabolites of these compounds.

Methods Employed: ^{14}C-N-Hydroxy-N-arylacetamides were synthesized following previously described procedures. Phenacetin N-O-glucuronide was synthesized by incubating Triton X-100 activated microsomes with N-hydroxyphenacetin and UDPGA. Phenacetin N-O-glucuronide was isolated by thin layer chromatography and incubated under varying conditions for 45 hrs. Metabolites were isolated by thin layer chromatography and quantitated by liquid scintillation spectrometry. All other methods were standard procedures.

Major Findings: After sulfation of N-hydroxyphenacetin and N-hydroxy-2-acetylaminofluorene, these substances decomposed to reactive metabolites and then immediately became covalently bound to protein. The N-O-glucuronide of N-hydroxyphenacetin similarly decomposed to a reactive metabolite but the rate of conversion was very slow; in Tris buffer at 37°C it had a half life of 8.7 hr. Four metabolites were formed: phenacetin, 2-hydroxyphenacetin glucuronide, acetaminophen and acetamide. In the presence of bovine serum albumin, covalent binding occurred at the expense of acetaminophen and acetamide. Glutathione (GSH) blocked the covalent binding and a GSH-acetaminophen conjugate was formed. With GSH, acetamide formation was blocked but acetaminophen formation was not. Ascorbic acid blocked covalent binding and acetaminophen formation was increased. It was concluded that phenacetin N-O-glucuronide decompose to the primary products: phenacetin, 2-hydroxyphenacetin glucuronide and N-acetylimidoquinone and that N-acetylimidoquinone combines covalently with protein, forms a GSH-acetaminophen, or undergoes hydrolysis to acetamide and quinone. The reactive metabolite produced after sulfation of N-hydroxyphenacetin was likewise postulated to be N-acetylimidoquinone since ascorbic acid blocked covalent binding and there was a concomitant increase in the formation of acetaminophen.

When phenacetin N-O-glucuronide was incubated in the presence of phosphate buffer an additional metabolite can be isolated: 3-hydroxyphenacetin phosphate. This metabolite was formed solely at the expense of acetaminophen, acetamide and covalent binding. Increasing concentrations of phosphate in the presence of protein revealed that at infinite phosphate concentration, covalent binding was only partially inhibited by phosphate, whereas acetaminophen and acetamide were completely inhibited. In the absence of protein, acetaminophen formation could not be completely inhibited by phosphate whereas acetamide formation could be completely inhibited by phosphate. These data indicated that three reactive intermediates were formed, two of which became bound to protein and one of which reacted with phosphate. The intermediate that reacted with phosphate was postulated to be the resonance stabilized nitrenium ion formed by heterolytic cleavage of the N-O-bond. In the absence of phosphate this intermediate would react with water to form the N-acetylimidoquinone, which in turn could bind to protein, undergo hydrolysis to acetamide plus benzoquinone, or be reduced to acetaminophen. The structure

of the other metabolite that binds to protein is unknown.

Significance to Biomedical Research and Program of the Institute: In this project we have elucidated mechanisms which may be important in the various toxicities of N-arylacetamides. These data also show the complexities associated with reactive metabolites.

Proposed Course of Project: Since studying the reactive metabolite produced by conjugation of N-hydroxyphenacetin offers a unique opportunity to examine metabolites which are important in tissue necrosis or cancer, the project will be continued.

Publications:

Mulder, G.J., Hinson, J.A. and Gillette, J.R.: Conversion of the N-O-glucuronide and N-O-sulfate conjugates of N-hydroxyphenacetin to reactive intermediate. Biochem. Pharm. 27: 1641-1649, 1978.

Hinson, J.A., Andrews, L.S., Mulder, G.J., and Gillette, J.R.: Decomposition mechanisms of N-O-glucuronide and N-O-sulfate conjugates of phenacetin and other arylamides in Conjugation Reactions in Drug Biotransformation, Ed. A. Aitio, Elsevier/ North-Holland Biomedical Press, p. 455-465, 1978.

Andrews, L.S., Hinson, J.A. and Gillette, J.R.: Studies on the mutagenicity of N-hydroxy-2-acetylaminofluorene in the Ames-Salmonella mutagenesis test system. Biochem. Pharm. 27: 2399-2408, 1978

Andrews, L.S., Fysh, J.M., Hinson, J.A. and Gillette, J.R.: Ascorbic acid inhibits covalent binding of enzymatically generated 2-acetylaminofluorene-N-sulfate to DNA under conditions in which it increases mutagenesis in Salmonella TA 1538. Life Sciences 24: 59-64, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00810-02 LCP

PERIOD COVERED
October 30, 1978 to September 30, 1979

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

PI:	Terrence Monks	Visiting Fellow	LCP	NHLBI
OTHER:	James R. Gillette	Chief, Lab. of Chem. Pharm.	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:
0.5	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)

Two theoretical models of hepatic drug clearance are being studied with respect to the formation and elimination of drug metabolites by the liver. One of the models is the "well-stirred" model in which it is assumed that the unbound concentration of the drug in blood and in all hepatocytes is the same. The other model is the "parallel tube" model in which it is assumed that the unbound drug concentration declines exponentially from the peripheral to the centrilobular region of the liver unit.

PHS-6040
(Rev. 10-76)

Project No. Z01 HL 00810-02 LCP

Project Description:

Objectives: The role of active and toxic metabolites in drug therapy is well recognized. Although the fate of a metabolite as formed from its precursor is usually assumed to be identical to that of the preformed metabolite, studies in previous years have indicated that the availability of the acetaminophen derived from phenacetin is slightly higher than that of preformed acetaminophen, indicating that the "well-stirred" model is not completely accurate in predicting steady-state concentration of metabolites that are rapidly cleared by the liver. The data also suggested that the availability was slightly less than that predicted by the "parallel-tube" model. However, the extraction ratio of phenacetin by rat liver is too large to permit an accurate assessment of the model. Other drug-metabolite pairs need to be studied.

Methods Employed: Radiolabeled tracer doses of drugs and their metabolites are infused simultaneously into liver preparations and the availabilities of the drugs and metabolites are measured.

Major Findings: The extraction ratio of radiolabeled acetanilide by liver is about 0.25. The formation of acetaminophen from acetanilide may thus provide a more accurate assessment of the heterogenous "parallel tube" model.

Significance to Biomedical Research and Program of the Institute: An understanding of the kinetics of metabolite formation and elimination by the liver should provide an assessment of the feasibility of using prodrugs to overcome the first-pass effects of liver.

Publications:

Pang, K.S. and Gillette, J.R.: Kinetics of metabolite formation and examination in the perfused rat liver preparation: Differences between the elimination of preformed acetaminophen and acetaminophen formed from phenacetin. Journal of Pharmacology and Experimental Therapeutics 207: 178-194, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00811-02 LCP

PERIOD COVERED

October 30, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Pharmacokinetic Aspects of Drug Disposition

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

PI:	James R. Gillette	Chief, Lab. of Chemical Pharm.	LCP	NHLBI
	Kim Chang Sandy Pang	Visiting Fellow	LCP	NHLBI

COOPERATING UNITS (if any)

Dr. Pang is on the Faculty of the University of Houston, College of Pharmacy, Houston, Texas

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Enzyme-Drug Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 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)

Pharmacokinetic equations for several theoretical models were derived: 1) Based on a single compartment for a drug and one of its primary metabolites several plotting techniques were invented for estimating the rate constant of elimination of the metabolite in the presence of the parent drug. 2) Plotting techniques were invented to evaluate the relative importance of decomposition pathways of chemically reactive metabolites. 3) Equations were derived for the area under the curve of a general model for drug disposition. 4) The meaning of the relative areas under the curves of drugs and their metabolites has been clarified.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Interpretations of *in vivo* and *in vtiro* experiments on drug disposition are frequently obscured by inadequate understanding of pharmacokinetic principles. The objective of this project is to derive equations for complex models that should aid in the design and interpretation of studies of drug disposition.

Methods Employed: Standard mathematical methods for multicompartmental analyses have been used.

Major Findings: 1) When the rate constant of elimination of a primary metabolite nearly equals or exceeds that of its precursor the rate constant of the metabolite cannot be evaluated from the apparent half-life of the metabolite formed from its precursor. Several plotting techniques based on ratios of the concentrations of the drug and its metabolite have been invented to estimate the difference between the rate constants. From the difference in rate constants and the direct measurement of the half-life of the parent drug, an estimate of the rate constant of elimination of the metabolite may be calculated.

2) The relative rates of reaction of chemically reactive metabolites with specific nucleophiles or reducing agents vary markedly. Studies on the effect of different concentrations of nucleophiles on the pattern of decomposition products may thus be used to evaluate the relative importance and complexity of the pathways of decomposition. In some cases the same product may be formed by several pathways. The plotting techniques we have invented greatly aid the estimation of the relative importance of these pathways.

3) Equations for the area under the blood concentration curves have been derived for a model in which a drug is eliminated by intestinal flora, intestinal mucosa, liver, lung, and kidney. These equations were based on the assumption that all organs could be viewed as "well-stirred" compartments. During the past year, we have converted these equations to model independent lineary systems based solely on mass balance equations. The equations show the interplay of blood flow rates, organ elimination, biliary excretion and intestinal blood to lumen diffusion in the disposition of durgs.

4) Equations derived for "well-stirred" compartment models in which drugs and their metabolites are eliminated solely by the liver reveal that the ratio of the areas under the curve for a primary metabolite and the parent drug after oral administration of the parent drug depend solely on the activities of the enzyme that catalyzes the formation of the metabolite and those that catalyze the elimination of the metabolite. The ratios are independent of the side reactions of the parent drug.

Significance to Biomedical Research and Program of the Institute: These pharmacokinetic models illustrate relationships which are not easily visualized by intuitive reasoning.

Proposed Course of Project: Equations for other models will be derived as needed.

Publications:

Gillette, J.R.: Bioactivation of nitroheterocyclic compounds to chemically reactive metabolites and superoxide. Metronidazole: Proceedings, Montreal, Excerpta Medica 20-22, 1978.

Pang, K.S. and Gillette, J.R.: Complications in the estimation of hepatic blood flow in vivo by pharmacokinetic parameters. Drug Metabolism and Disposition 6: 567-576, 1978.

Pang, K.S. and Gillettec J.R.: A theoretical examination of the effects of gut wall metabolism, hepatic elimination, and entero-hepatic recycling on estimates of bioavailability of hepatic blood flow. Journal of Pharmacokinetics and Biopharmaceutics 6: 355-367,

Gillette, J.R.: Chairman's Introductory Statement: Prenatal development of drug metabolism. In The Induction of Drug Metabolism (Eds. R. Estabrook & E. Lindenlaub) pp. 501-506, 1979.

Gillette, J.R.: Toxicological implications of drug metabolism. In The Induction of Drug Metabolism (Eds. R. Estabrook & E. Lindenlaub) pp 309-325, 1979.

Gillette, J.R.: Biotransformation of drugs during aging. Federation Proceedings 38: 1900-1909, 1979

Honors and Awards:

Bernard B. Brodie Award in Drug Metabolism - 1978
Doctor of Science, Cornell College, Mt. Vernon, Iowa, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00831-04 LCP

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

Chloramphenicol-induced Aplastic Anemia

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

PI:	Gopal Krishna	Chief, Sect. on Drug-Tissue Inter.	LCP	NHLBI
	Ibrahim Aykac	Guest Worker	LCP	NHLBI
OTHERS:	Guruva B. Reddy			FDA
	Dr. R. Killens	Chief, Comparative Medicine Unit	VR,	DRS.
	Upton Talley	Biol. Lab. Tech. (animals)	OD	NHLBI

COOPERATING UNITS (if any)

Veterinary Research Branch, Divisional Research Service, NIH, FDA

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Section on Drug-Tissue Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	0.8	1.2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Both chloramphenicol and thiamphenicol-induced servere aplastic anemia in bovine calves when administered orally at a dose of 100 mg/kg/day for 10 days. Aplastic anemia induced by thiamphenicol was completely prevented by co-administration of B complex vitamins, Vit. B12 and folic acid. However, chloramphenicol-induced aplastic anemia was only reduced and not completely prevented by the administration of vitamin mixture indicating a still unknown mechanism is involved in chloramphenicol induced aplastic anemia.

Project Description

Objectives: During the past couple of years we have been investigating the role of drug metabolism in chloramphenicol induced aplastic anemia in calves. Last year we compared the incidence of aplastic anemia caused by chloramphenicol and thiamphenicol after oral and intravenous administration into calves. We found the incidence of toxicity produced by these drugs was markedly lower after intravenous administration even though the drug levels in plasma were 50-100 times greater than those obtained when the drugs were administered orally. Pharmacokinetic calculations suggested that only 2-4% of the oral dose was bioavailable. This finding led us to believe that the drugs might be converted by the microflora in the stomach to reactive metabolites which might cause the disease. We also checked the alternative possibility that the drugs might interfere with the microfloral production of nutrients needed for the animal by coadministrating B complex vitamins, vitamin B_{12} and folic acid.

Methods Employed: About 20 Holstein calves weighing 80-100 kg have been utilized for this study. The calves were fed ad libitum cow chow and had free access to water. Blood samples were collected daily for 5 days before the experiment. A total blood cell count was performed. Chloramphenicol or thiamphenicol (100 mgs/kg/day) was administered orally in gelatine capsules for 10 days. Another group of animals not only received the drug orally but also were administered (i.v.) a mixture of B complex vitamins containing folic acid (5 mg) and vitamin B_{12} (0.5 mg) daily for a 20-day period (10 days during the drug treatment and 10 days post). Blood samples were collected at weekly intervals. The animals were sacrificed at the end of 6 weeks. Towards the end of the experiment biopsies of the bone marrow were obtained by sternal puncture, and the bone marrow smears were fixed immediately with methanol and stained as described below. Necropsies were performed on each aniaml in search of gross abnormalities of internal organs. Bone marrow from ribs, sternum and from femoralhaed were collected and fixed in buffered formalin or methanol. Sections of lymph node, liver, spleen and kidney, thymus and adrenals were fixed in methanol, Giemsa Wright, May Greenwald Giemsa for cellular morphology and brilliant creosol for reticululocyte vital staining. Bone marrow smears were fixed in methanol and stained as above for the study of cytological characteristics of the bone marrow cells. Bone marrow cells as well as other tissue sections were also stained for the presence of free iron. Methyl green pyronin was used for staining RNA, DNA and their precursors in blood cells. In order to differentiate lymphocytes from other leucocytes, histochemical staining for peroxidase was employed. Cytoplasmic or nuclear vacuolization due to oil droplets in bone marrow cells was evaluated by staining the bone marrow or blood smear with Sudar IV or oil Red O.

Paraffin sections of bone marrow were stained for peroxidase, DNA, RNA and Free iron. Sections of other tissues were stained with hemotoxylin and eosin.

Major Findings: On necropsy of the animals which had been treated with

chloramphenicol or thiamphenicol, no gross lesions were revealed in the thoracic or abdominal organs except for some hyperemia in the sinus membranes. Peripheral blood smears obtained from treated animals revealed a predominance of lymphocytes in comparison to untreated animals.

The proerythroblasts in the blood marrow smears and sections had vacuoles in the nucleus and cytoplasm. They were extensive aplasia in the bone marrow sections of animals treated with chloramphenicol or thiamphenicol. Tissues like kidney, liver, spleen, lymph node showed a high degree of haemorrhage. The changes in the bone marrow were induced by thiamphenicol were completely prevented by co-administration of vitamin mixture. The bone marrow appeared almost normal. In case of animals treated with chloramphenicol the bone marrow changes were reduced but not aliminated by administration of vitamin mixture. The extensive aplasia of the bone marrow induced by chloramphenicol was reduced to some extent by treatment with vitamin mixture but these were large areas of focal aplasia in the bone marrow. Administration of vitamin mixture appeared to reduce further the lower incidence of aplastic anemia induced by intravenous administration of thiamphenicol.

Significance to Biomedical Research and Program of the Institute: It appears from these studies that the mechanism of aplastic anemia induced by chloramphenicol may be different from that induced by thiamphenicol. Even though treatment with vitamin mixture afforded a complete protection from thiamphenicol induced aplastic anemia, it does not afford a similar protection in case of aplastic anemia induced by chloramphenicol. It is possible a part of the mechanism of aplastic anemia is related to the inhibition of microflora in the stomach which appear to be essential in providing vitamin requirement for the animal. In case of chloramphenicol there appears to be a still unknown mechanism responsible for a part of aplastic anemia induced by this drug.

Proposed Course of Project: We propose to investigate whether chloramphenicol induced aplastic anemia by being converted to various nitro reduction products by the microflora in the stomach. We plan to investigate by studying the nitroreduction of chloramphenicol by these mecroorganisms as well by investigating the capactiy of various nitro reduction products of chloramphenicol to induce aplastic anemia in calves and in laboratory animals.

Publicatons: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00832-04 LCP

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
The Role of Anacardic Acid in Platelet Function

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

PI:	Gopal Krishna	Chief, Sect. on Drug Tissue Interaction	LCP	NHLBI
	Nancy Kim	Chemist	LCP	NHLBI
	Helen Lloyd	Chemist	LC	NHLBI

COOPERATING UNITS (if any)
Laboratory of Chemistry, NHLBI

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Drug-Tissue Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Anacardic acid, a salicylic acid analog, which is a constituent of cashew nut shell, inhibited markedly the increase in cyclic AMP (cAMP) induced by prostacyclin (PGI_2) in human blood platelets. It also inhibited markedly the synthesis of thromboxane B_2 (TXB_2) from ^{14}C-arachidonic acid. Anacandic acid did not inhibit platelet aggregation induced by arachidonic acid but overcame the inhibition induced by PGI_2. These findings indicate that anacardic acid may interact with PGI_2 receptors in blood platelets and may be useful in controlling platelet functions.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Anacardic acid is present in the shells of cashew nut to the extent of 85%. It is a derivative of salicylic acid containing an alkyl group of 15 carbons attached at the 6 position of salicylic acid and contains 1, 2, or 3 double bonds depending on the origin of the cashew nut. So far very little is known regarding its pharmacological action. Since it represents an unusual analog of salicylic acid we have examined its effect on the human platelet function.

Methods Employed: Human blood was collected in dextrose-citrate buffer and platelet-rich plasma was prepared by differential centrifugation. Platelet aggregation was measured in the Bio-Data Aggregometer. Cyclic AMP (cAMP) was measured in platelet rich plasma in response to various concentrations of prostacyclin (PGI_2) in the presence and absence of various analogs of anacardic acid. The synthesis of thromboxane (TXB_2) and other prostaglandins was measured in washed platelet suspensions incubated with ^{14}C-arachiodonic acid. TXB_2 and prostaglandins were fractionated by chromatographic systems as reported last year. Anacardic acid was extracted from the cashew nut shells with methanol, isolated as lead salt and the various analogs of anacardic acid were then separated by high pressure liquid chromatography on a preparative reverse phase column. All the fractions have been characterized for purity by various chromatographic systems and the structures have been elucidated by GC-Mass Spectroscopy.

Major Findings: Anacardic acid inhibited markedly cAMP formation induced by PGI_2. The inhibition appears to be competitive in that high concentrations of PGI_2 were able to overcome the inhibition produced by a low concentration of anacardic acid. Salicylic acid at similar concentrations did not inhibit cAMP formation. Preliminary experiments indicated that the triene analog of anacardic acid appears to have greater inhibitory effect on cAMP formation in platelets than does the monoene analog.

Anacardic acid inhibited markedly (90%) of the TXB_2 synthesis in washed human platelet suspensions. At low concentrations anacardic acid inhibited TBX_2 synthesis to a greater extent than it inhibited the synthesis of other prostaglandins. The inhibition of TXB_2 and prostaglandin synthesis is competitive with arachidonic acid.

Anacardic acid did not aggregate platelets nor did it inhibit the aggregation induced by 0.5 mM arachidonic acid. However, anacardic acid decreased the inhibition of aggregation produced by PGI_2. In a preliminary study, it appears that the triene analog of anacardic acid had a greater effect than the monoene analog. Whether these analogs of anacardic acid produces the inhibition by competing for the PGI_2 receptor in platelets is not known but this appears to be a possibility. This will require a direct approach utilizing labeled PGI_2 in order to study the interaction of the PGI_2 receptor with anacardic acid.

Significance for Biomedical Research and Program of the Institute: Finding the analog of salicylic acid which shows a specific interaction with PGI_2 receptor and which has marked inhibitory effect on TXB_2 synthesis may aid in the understanding of the various parameters involved in platelet function and thrombosis.

Proposed Course of Project: We propose to study in detail the interaction of various analogs of anacardic acid on PGI_2 receptor in platelets and to delineate the mechanism involved in inhibition of TXB_2 synthesis.

Publications:

Giri, S.N. and G. Krishna. The effects of paraquat on prostaglandin synthesis of guinea pig lungs. Toxicology 11: 345-351, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00834-04 LCP

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Cyclic 3'5' Cytidine Monophosphate

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

PI:	Gopal Krishna	Chief, Sect. on Drug-Tissue Inter.	LCP	NHLBI
OTHER:	Thomas Hundley	Bio. Lab. Tech.	LCP	NHLBI

COOPERATING UNITS (if any)

Dr. F.X. Cole, Collabarative Research Inc. Waltham, Mass.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Drug-Tissue Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Very sensitive radioimmunoassay (RIA) for cyclic 3'5'-cytidine monophosphate (cCMP) has been developed using a very specific antisera raised against cCMP in rabbits. The RIA for cCMP is capable of measuring less than 10 femntomoles of cCMP. Since cyclic 3'5'-adenosine monophosphate (cAMP) cross reacts in this assay system, cAMP is separated from cCMP by chromatography Dowex 1-formate and small aliquots are collected in order to avoid interference of cAMP in cCMP peak. With this method, it has not been possible to show the presence of cCMP in rat liver, human urine or mouse leukemia L1210 cells. Rat pancreas is the only tissue which appear to contain cCMP 0.5-1 pmole/g. It requires further confirmation. Whether cCMP plays a role in cell proliferation has yet to be established.

Project Description:

Objectives: Last year we reported that we were unable to detect cytidylate cyclase, which converts CTP to cyclic 3'5' cytidine monophosphate (cCMP) in various tissues including mouse leukemia cells (L1210) either grown in peritonial cavity of mice or in tissue culture. Previously, it has been reported that cCMP is present in large concentrations in mouse leukemia L1210 cells and it may play an important role in cell proliferation. In order to determine whether very high concentrations of cCMP are found in rapidly dividing cells such as mouse leukemia L1210 cells, it was essential to develop a specific radioimmunoassay for cCMP in tissues.

Methods Employed: ^{125}I-cCMP antigen and antisera were prepared by a procedure similar to that developed for cAMP and cGMP radioimmunoassay systems. cCMP was isolated from various rat tissues and L1210 cells by homogenization with 6% trichloracetic acid. The tissue extracts after removal of TCA with diethyl ether were chromatographed on a Dowex 1-formate column (Bio Rad AG1-X-8, 200-400 mesh, 0.5 x 4 cm). The columns were eluted with increasing concentrations of formic acid; 2 ml aliquots were collected, lyopholized and dissolved in sodium acetate buffer (pH 6.2) for RIA for cCMP and cAMP. RIA of cCMP was preformed in a similar fashion as described in earlier reports for cAMP and cGMP, after succinylation of the nucleotide.

Major Findings: So far we have obtained six antisera exhibiting very high affinities towards cCMP but very low cross activity towards all other cyclic nucliotides. Only cAMP appears to cross react with the antisera to an extent that it may intefere with RIA of cCMP when cCMP is not removed by Dowex 1-chromatography. Thus it is essential to collect small fractions for the assay of cCMP in order to prevent the interference of cAMP in the assay of tissues containing large concentrations of cAMP. However, it is possible to assay less than 10 femntomoles of cCMP by the antisera that had the highest affinity towards cCMP.

Even though the assay system for cCMP appears to be very sensitive, we have so far not been able to detect cCMP in rat liver, human urine or mouse leukemia L1210 cells which were either grown in tissue culture or in the peritonial cartilage of mice. Rat pancreas appear to be the only tissue that contain cCMP. Since the content of cCMP is very low, only 0.5-1 pmole/g, further confirmation is necessary to prove the existence of cCMP in this tissue. Earlier reports from other laboratories indicating the presence of very large concentration of cCMP or the enzyme responsible for its synthesis in L1210 cells in other tissues have not been confirmed by our studies. The possible role of cCMP in cell proliferation remains to be studied.

Significance to Biomedical Research and Program of the Institute. cCMP may not be present in rat liver and mouse leukemia L1210 cells to the extent claimed by other laboratories mainly because of the interference of cAMP in the cCMP assays. If the cCMP presence in the rat pancreas is

confirmed by further studies, it may be the only tissue that contains measurable amounts of cCMP.

Proposed Course of Project: We propose to establish whether cCMP is present in rat pancreas and to examine the tissue for the presence of enzyme system for the synthesis of cCMP. If cCMP is normally present in pancreas it may be possible to examine whether it is increased in certain pancreatic tumors or involved in pancreatic secretion in response to hormone.

Publications:

Gaion, R.M. and Krishna, G.: Cytidylate cyclase: The product isolated by the method of Cech and Ignarro is not cytidine 3',5'-monophosphate. Biochem. Biophys. Res. Commn. 86: 105-111, 1979.

Gaion, R.M. and Krishna, G.: Cytidylate cyclase: Possible artifacts in the methodology. Science 203: 672-673, 1979.

Kapoor, C.L. and Krishna, G.: A possible role for guanosine 3',5'-monophosphate in the stimulus-secretion coupling in exocrine pancreas. Biochim. et Biophys. Acta 544: 102-112, 1978.

Liu, Y.P., Krishna, G., Aguirre, G. and Chader, G.J.: Cyclic GMP phosphodiesterase activator: Involvement in a hereditary retinal degeneration. Nature 280: 62-64, 1979.

Kapoor, C.L. and Krishna, G.: Noncompetitive inhibition of soluble guanylate cyclase by 2'-deoxyguanosine-3'-monophosphate. Biochemical Pharmacology, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00835-03 LCP

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Investigation of the Mechanism of Activation of Chloroform

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

PI:	Lance R. Pohl	Sr. Staff Fellow	LCP	NHLBI
OTHERS:	Jackie L. Martin	Chemist	LCP	NHLBI
	John W. George	Chemist	LCP	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme-Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.1	0.7

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Chloroform ($CHCl_3$) a hepatotoxin, has previously been shown to be oxidatively dechlorinated to phosgene ($COCl_2$) by cytochrome P-450 in rat liver microsomes. We have further characterized the metabolism of $CHCl_3$ in rat liver microsomes by measuring the covalent binding of [^{14}C] $CHCl_3$ and [^{3}H] $CHCl_3$ to microsomal protein as well as the formation of chloride ion, $COCl_2$ and CO_2 under a variety of conditions. The results further confirm that $CHCl_3$ is oxidatively dechlorinated to $COCl_2$ by liver microsomal. In addition, a form of cytochrome P-450 that is induced by phenobarbital appears to catalyze this reaction. Once formed, $COCl_2$ either covalently binds to microsomal protein with the release of chloride or undergoes hydrolysis to form CO_2 and chloride. $CHCl_3$ does not appear to be activated by reductive dechlorination to $CHCl_2$ radical because the [^{3}H] label did not bind to microsomal protein. These findings not only define more clearly the mechanism of metabolic activation of $CHCl_3$, but also should serve as a model for determining the pathways biotransformation of other halocarbon environmental chemicals and drugs.

Project Description:

Objectives: We previously reported that chloroform was biotransformed by rat liver microsomes into phosgene ($COCl_2$) and CO_2. The objective of the present investigation has been to more fully characterize the mechanism of bioactivation of $CHCl_3$.

Methods Employed: [^{3}H] labeled $CHCl_3$ was synthesized by an exchange reaction with an alkaline solution of tritiated water. The metabolism of $CHCl_3$ in liver microsomes was studied by measuring the covalent binding of [^{14}C] $CHCl_3$ and [^{3}H] $CHCl_3$ to microsomal protein as well as the formation of chloride ion, CO_2 and $COCl_2$ (assayed as its cysteinyl derivative).

Major Findings: The conversion of [^{14}C] $CHCl_3$ to covalently bound [^{14}C] label and to chloride, $COCl_2$ and CO_2 was maximal when the incubations were conducted in air. Parallel decreases in each of these substances occurred when the reactions were performed in atmospheres of N_2 or $CO:O_2$, or when NADPH was absent or SKF 525A was present in the reaction mixtures. In contrast, insignificant amounts of the [^{3}H] label of [^{3}H] $CHCl_3$ were bound to liver microsomal protein under all of the incubation conditions.

The amount of $COCl_2$ produced in microsomes from phenobarbital pretreated rats was approximately 10 times higher than the level formed in untreated microsomes, whereas the amount produced in 3-methylcholanthrene enzymes was slightly lower than that of the untreated preparations.

Pretreatment of animals with phenobarbital significantly increased the hepatoxicity of $CHCl_3$ as compared to the untreated rats while pretreatment with 3-methylcholanthrene had an insignificant potentiating affect.

Significance to Biomedical Research and Program of the Institute: These results further indicate that the major pathway of metabolism of $CHCl_3$ in liver microsomes is through an oxidative dechlorination to $COCl_2$. Once produced, $COCl_2$ would be expected to either covalently bind to microsomal protein with the concomittant release of Cl ion or hydrolyse to CO_2 and Cl ion. The parallel increases and decreases in the levels of $COCl_2$, Cl^-, CO_2, and [^{14}C] label binding under the various incubation conditions supports this intermediate role of phosgene. In addition, the formation of $COCl_2$ appears to be catalyzed by a form of cytochrome P-450 that is induced by phenobarbital and not by a form that is induced by 3-methylcholanthrene. This inducible form of cytochrome P-450 also appears to potentiate the hepatotoxicity of $CHCl_3$ by presumably increasing the rate of formation of $COCl_2$ in the liver. $CHCl_3$ does not appear to be activated by a reductive dechlorination to $CHCl_2$ radical because the [^{3}H] label did not bind to microsomal protein *in vitro*.

The oxidative dehalogenation mechanism outlined for $CHCl_3$ may have general importance, since other toxic halocarbons such as chloramphenical, halothane, enflurane and methoxyflurane may be metabolized by a similar

mechanism. This possibility can be investigated by studying the bioactivation of [^{14}C] and [^{3}H] labeled derivatives of these compounds.

Proposed Course of Project: Further in vitro studies on the metabolic activation of $CHCl_3$ will be conducted in order to characterize more completely the pathways of its metabolism. In particular, we plan to study the metabolic activation of [^{36}Cl] labeled $CHCl_3$ in liver microsomes. If this label binds covalently to microsomal protein, then $CHCl_3$ may be activated by another mechanism as well as by oxidative dechlorination to $COCl_2$. We also plan to determine whether $CHCl_3$ is activated to free radicals in microsmes by monitoring the reaction with electron spin resonance spectrometry. Such studies should increase our understanding of the mechanisms by which liver microsomes activate $CHCl_3$. These findings should serve as a model for defining the mechanisms of metabolic activation of other halocarbon compounds by liver microsomal enzymes.

Publicatons:

Pohl, L.R.: Biochemical Toxicology of Chloroform: Reviews in Biochemical Toxicology, Eds. Hodgson, Bend, and Philpot, Elsevier North Holland, Inc., pp. 79-107, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00837-03 LCP

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

On the Mechanism of Hydrolytic Dechlorination of Chloramphenicol

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

PI:	Lance R. Pohl	Sr. Staff Fellow	LCP	NHLBI
OTHERS:	Jackie L. Martin	Chemist	LCP	NHLBI
	John W. George	Chemist	LCP	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme-Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.2	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)

Chloramphenicol ($RNHCOCHCl_2$) undergoes dechlorination to form two products by a glutathione dependent enzyme or enzymes in 100,000 x g supernatant of the rat liver. These metabolites have been identified by gas chromatography mass spectrometry as CAP aldehyde (R-NHCOCOH) and a CAP oxamyl derivative (R-NHCOCO-) which is believed to be a GSH thioester.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: We previously reported that chloramphenicol (CAP) was metabolized into 2 metabolites by a glutathione dependent enzyme or enzymes in the 100,000 x g supernatant (cytosol) of rat liver. The objective of this investigation has been to characterize more completely the structure of these metabolites and the pathways leading to their formation.

Methods Employed: Radiolabeled derivatives of CAP were incubated with the cytosol fraction of rat liver for 1 hour. The reaction mixture was then extracted with ethyl acetate at various pH values and the extracted metabolites were purified by HPLC and derivatized and characterized by gas chromatography mass spectrometry. The effect of pretreatment of rats with inducers of liver enzymes on the formation of these metabolites was also investigated.

Major Findings: The least polar metabolite was extracted into the organic phase when the reaction mixtures at pH 7 was shaken with ethyl acetate. The extract was reduced with sodium borodeuteride to give a product with the same HPLC retention time and GCMS characteristics as CAP alcohol. Since only one deuterium was incorporated into the product, this established that the original metabolite was CAP aldehyde (RNHCOCHO), formed by the replacement of the 2 chlorine atoms of CAP ($RNHCOCHCl_2$) by an oxygen atom. A polar metabolite was not extracted into ethyl acetate from neutral or acidic aqueous solution. However, when the pH of the aqueous solution was increased to pH 10 for 15 minutes, followed by acidification to pH 1 with HCl, the polar metabolite was extracted into ethyl acetate. This compound was characterized as CAP oxamic acid (RNHCOCOOH) by reverse isotope dilution, chemical conversion to methyl CAP oxamate and by GCMS. These results indicate that the polar metabolite is a derivative of CAP oxamic acid which is readily hydrolysed to CAP oxamic acid at pH 10. It is likely that the metabolite is a GSH thioester, since its formation depends upon the presence of GSH and because a GSH thioester of CAP oxamic acid would be expected to undergo hydrolysis at pH 10.

Pretreatment of rats with phenobarbital or 3-methylcholanthrene do not alter the rate of formation of the 2 metabolites.

Significance to Biomedical Research and Program of the Institute: These results establish that CAP is dechlorinated into an aldehyde and an oxamyl derivative. These reactions may represent general pathways for the metabolism of other halocarbons. Since the metabolites of these reactions would be more water soluble than the parent drug, they should be readily excreted into urine. Consequently, these pathways of metabolism may represent detoxifying processes. However, it is possible that the aldehyde and acyl derivative metabolites may have pharmacologic or toxicologic properties.

Proposed Course of Project: We plan to establish the structure of the CAP oxamyl derivative. In addition, we intend to characterize more

completely the enzymes which metabolize CAP into CAP aldehyde and the oxamyl derivative. These studies will also be extended to halocarbon drugs such as dichloroacetic acid, halothane, thiamphenicol, and enflurane, in order to determine whether these are metabolized by the enzyme.

Publications:

Pohl, L.R., Reddy, G.B. and Krishna, G.: A new pathway of metabolism of chloramphenicol which influences the interpretation of its irreversible binding to protein *in vivo*. *Biochemical Pharmacology*, in press, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 00840-03 LCP

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

Effect of Chemical Modification of Diphenylhydantoin on its Toxicity

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

PI:	Bharat Bhooshan	Guest Worker	LCP	NHLBI
	Gopal Krishna	Chief, Sect. on Drug-Tissue Inter.	LCP	NHLBI
OTHERS:	A.L. Wilk	Chemist	LCBA	NIDR

COOPERATING UNITS (if any)

Dr. A.L. Wilk is a chemist in the Laboratory of Developmental Biology and Anomalies, NIDR.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Section on Drug-Tissue Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Seven analogues of diphenylhydantoin (DPH) synthesized during the last two years, were tested for their capactiy to induce cleft palate and cleft lip in two species of mice. All analogues of DPH except monomethylphenyl analog of DPH induced a high incidence of cleft palate and cleft lip in NIH strain or A/JAX mice. All analogues of DPH, except the dihalo derivatives were metabolised by hydroxylation of the phenyl groups and excreted in the urine as glucuronyl conjugates in both species of mice. Even though difluro analogue is not metabolised, and excreted in the urine as unchanged drug, it induced a very high incidence of cleft palate in both species. This finding indicates that metabolism of DPH by hydroxylation (epoxidation) pathway may not be a pre-requisite for the induction of cleft palate.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Dihenylhydantoin [DPH] is commonly used for the treatment of epilepsy in children. However, it causes a number of serious side effects. DPH has been known to induce gingival hyperplasia in children and cleft palate and cleft lip in infants when the mother is on DPH medication. The mechanisms by which these drug toxicities are produced are unknown. Since many other drugs have been shown to induce various toxicities through metabolic activation to reactive intermediates, we have examined a number of analogues of DPH, which are metabolised less rapidly than DPH, for their capacity to induce cleft palate and cleft lip in mice.

Methods Employed: Various analogues of DPH were prepared as described in 1976-1977 report. Female albino mice (A/JAX and NIH strain), were coupled in the laboratory and the pregnancy of each animal was confirmed by vaginal smears. DPH and its analogues were dissolved in a solvent consisting of 1N NaOH containing 10% ethanol and 40% propyleneglycol. Groups comprising 5-10 pregnant mice were injected intramuscularly on the 9-12th day of gestation with drugs at 75, 100, and 150 mg/kg dose levels. Control animals received a similar amount of the solvent.

The animals were sacrificed on the 23rd day of gestation. The uterus was removed and the number of resorptions was noted. The fetuses were removed and examined for external malformation, fixed in Bouins fluid for 2-3 weeks, and examined for cleft palate and cleft lip by microscope.

Major Findings: The results on A/JAX mice are summarized in Table 1. At a dose of 75 mg/kg DPH produced an incidence of 83% cleft lip-cleft palate (CL/CP) when injected on the 10th day of gestation and 12-14% of incidence when it was injected on the 9th or 11th day of gestation. The monofluoro derivative produced an incidence of 20% CP and 40% CL/CP when injected on 10th day of gestation and no incidence when injected on the 9th day. Similarly difluoro derivative of DPH produced a very high incidence (85%) when injected on the 10th day and a lower incidence (27%) when injected on the 9th day of gestation. The decadeutero derivative of DPH induced a very high incidence (86%) of CL/CP when injected on the 10th day while pentadeutero derivative induced a much lower incidence (15%) of CL/CP. The methyl derivative of DPH did not cause any incidence of CL/CP in A/JAX mice.

Similar studies were conducted in the NIH strain (general purpose) mice. The results are summarized in Table 2. DPH induced a dose dependent cleft palate (CP) formation when administered on the 11th day of gestation. DPH induced 80% incidence of CP at a dose of 150 mg/kg im. on the 11th day while inducing only a 13% when administered on the 10th day of gestation. The monofluoro derivative of DPH induced a high incidence of cleft palate when administered on the 10th day rather than the 11th day (62% vs. 10%). Difluoro analog also induced a high incidence CP when injected on the 10th day rather than on the 11th day (83% vs. 34%). The monochloro derivative of DPH induces at higher incidence (76%) of CP than dichloro derivative (6%) when administered

on the 11th day of gestation. The penta and deca deutero analogues of DPH also induced a very high incidence of CP when administered on the 11th day (84% and 83%). The methyl analogue of DPH caused only a small incidence of CP (27%) when administered on the 11th day of gestation. The monofluoro and difluoro are able to induce a very high incidence of CP even when administered on the 10th day of gestation when DPH induces very low incidence of CP. Difluoro analogues of DPH appear to be excreted in the urine unchanged and no metabolite have been detected by gas chromatography-mass spectroscopy. The monofluoro analogue appear to be metabolized by hydroxyation of one of the phenyl ring. There appear to be no major defluoroination reaction to account for the formation of DPH from these fluorinated derivatives of DPH. It thus appears that CP/CL induced by these DPH derivatives may be related to the unmetabolized drug rather than the formation of any epoxide metabolite.

Significance to Biomedical Research and Program of the Institute: It appears from these studies that hydroxylation of the phenyl ring in DPH may not be necessary for the induction of cleft palate-cleft lip in mice.

Proposed Course of Project: These derivatives of DPH will be examined further in the ferret model to examine if metabolism plays any role in the induction of gingival hyperplasia.

Publications: None

Compound	Dose mg/Kg	Gestation Day of Injection	Number of						%		
			Mothers	Mothers Died	Young	R[1]	CP[2]	CL/CP[3]	CP[2]	CL/CP[3]	R[1]
DPH	75	9	8	1	37	40	5	6	13	14	52
	75	10	6	0	40	11	0	33	0	83	21
	75	11	5	0	42	6	8	5	19	12	12
DPH-F	75	10	5	1	30	13	6	12	20	40	30
	75	9	5	0	33	13	0	0	0	0	28
DPH-F_2	75	10	4	2	13	16	11	0	85	0	55
	75	9	3	0	15	18	4	1	27	7	54
DPH-d_5	75	10	2	0	13	3	0	2	0	15	19
DPH-d_{10}	75	10	2	0	7	5	0	6	0	86	42
DPH-CH_3	75	10	4	0	18	9	0	0	0	0	33

[1] Resorption, [2] Cleft-palate, [3] Cleft-lip /Cleft-palate

Table I

Compound	Dose mg/Kg	Gestation Day of Injection	Number of					%	
			Mothers	Mothers Died	Young	R[1]	CP[2]	CP[2]	R[1]
DPH	150	10	3	0	16	13	2	13	45
	150	11	9	2	44	37	35	80	46
	100	11	10	0	75	11	35	47	13
	75	11	4	1	36	0	1	3	0
	150	12	11	4	84	18	39	46	17
DPH-F	150	10	2	1	13	5	8	62	28
	150	11	4	2	20	10	2	10	33
	100	11	8	1	23	46	13	57	67
	75	11	6	0	46	2	1	2	4
DPH-F_2	150	10	2	1	18	1	15	83	5
	150	11	11	0	68	36	5	74	35
DPH-d_5	150	11	5	3	33	16	31	94	33
	100	11	8	3	38	30	6	58	44
	75	11	6	0	42	7	2	5	14
DPH-d_{10}	150	11	3	3	6	20	5	83	77
	100	11	10	1	50	34	42	84	41
	75	11	9	1	63	6	0	0	9
DPH-Cl	150	11	8	5	41	38	31	76	48
DPH-Cl_2	150	11	10	3	67	24	4	6	26
DPH-CH_3	150	11	8	1	85	3	23	27	3

[1] Resorption, [2] Cleft-palate

Table II

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00851-06 LCP

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Paraquat Toxicity in Rat and Mouse

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

PI:	Harriet M. Maling	Chief, Section on Physiology	LCP	NHLBI
Other:	James R. Gillette	Chief, Lab. of Chem. Pharm.	LCP	NHLBI
	Wilford Saul	Chemist	LCP	NHLBI

COOPERATING UNITS (if any)
Dr. William J. Waddell, Prof. of Pharmacology, Univ. of Louisville, Health Sciences Center, Louisville, Ky. 40201

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Physiology

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The potent hydroxyl radical scavenger, 1-phenyl-3-(2-thiazolyl)-2-thiourea (PTTU) (50, 100,150, and 200 mg/kg i.p. 1 hr before paraquat) failed to prevent deaths in mice injected with paraquat (30, 35 and 40 mg/kg i.p.). Cellular resolution autoradiography of lungs removed at 3, 24 and 48 hr after intravenous injection of (methyl-^{3}H) paraquat chloride into male C57Bl/6J mice indicated that the radioactivity within the lung was confined almost entirely to the alveolar type II cells.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The toxicity of paraquat could result from the highly reactive hydroxyl radical, which may be formed by the Haber-Weiss reaction from the superoxide free radical and hydrogen peroxide. If this were the mechanism of paraquat toxicity, the highly potent hydroxyl radical scavenger, PTTU (1-phenyl-3-(2-thiazolyl)-2-thiourea) should protect mice against death from paraquat. Experiments were designed to test this possibility.

Cellular resolution autoradiography was undertaken to determine whether paraquat was taken up preferentially by special cells within the lung.

Methods Employed: Standard methods were employed.

Major Findings: Pretreatment with the potent hydroxyl radical scavenger, PTTU, does not protect mice from death induced by paraquat.

Cellular resolution autoradiography of lungs removed from rats 3, 24, and 48 hr after intravenous injection of [methyl-^{3}H] paraquat chloride indicates that the radioactivity within the lung was greatest in the alveolar type II cells.

Significance to Biomedical Research and Program of the Institute: Preferential uptake and localization of paraquat in type II cells justified rough calculations of intracellular concentrations of paraquat, based on tissue levels of paraquat. We have previously reported concentrations of about 15 nmole/gram lung in rats 6 hr after i.p. injection of 15 mg/kg paraquat. If all the lung paraquat were localized within type II cells and if 10% of the lung tissue consists of type II cells, then the concentration of paraquat within type II cells should be about 150 nmole/gram or about 1.5×10^{-4}M. Such calculations should facilitate correlations of tissue levels of paraquat with intracellular concentrations and concentrations needed in *in vitro* experiments to produce definite effects.

Proposed Course of Project: The results with autoradiography will be prepared for publication.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00852-03 LCP

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Isoproterenol on "Average Total Body Clearance" of Various Compounds in Rats

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

PI:	Harriet M. Maling	Chief, Section on Physiology	LCP	NHLBI
Other:	Wilford Saul	Chemist	LCP	NHLBI
	Wilson J. Yasaka	Visiting Fellow	LCP	NHLBI
	James R. Gillette	Chief, Lab. of Chem. Pharm.	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:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

In the conscious rat, l-isoproterenol (0.3 mg/kg s.c.) decreased about 50% both the glomerular filtration rate (GFR) and the renal plasma flow (RPF), as estimated by the total body clearance of ^{3}H-methoxyinulin and ^{14}C-tetraethyl-ammonium. This reduction in GFR and RPF may be the mechanism responsible for the potentiation of toxicity of paraquat and other compounds which are rapidly excreted by the kidney in an unchanged form. Pretreatment with the beta adrenergic blocking agent, propranolol, prevented the decrease by isoproterenol in GFR and RPF. These effects of isoproterenol were not mediated by release of antidiuretic hormone from the posterior pituitary since they were as great in hypophysectomized as in sham-operated rats. Peak plasma concentrations of isoproterenol after subcutaneous injection (0.3 mg/kg) in intact male Sprague-Dawley rats were about 40 ng/ml.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The purpose of this project is to explain the potentiation by large doses of l-isoproterenol (0.3 mg/kg s.c.) of the toxicity of paraquat and a number of compounds which are rapidly excreted by the kidney in an unchanged form.

Methods Employed: GFR and RPF were estimated from the simultaneous measurements of the total body clearances of ^{3}H-methoxyinulin and ^{14}C-tetraethylammonium, using the relationship Cl = Dose i.v./AUC.

Major Findings: A large dose of l-isoproterenol (0.3 mg/kg s.c.) reduced both the GFR and RPF about 50%. This reduction in GFR and RPF may be responsible for the potentiation of toxicity of compounds rapidly excreted by the kidney in an unchanged form. These effects of l-isoproterenol are probably beta adrenergic effects, since they were prevented by pretreatment with the beta adrenergic blocking drug, propranolol. Since the decrease in GFR and RPF were as marked in hypophysectomized rats as in sham-operated rats, these effects do not require release of the antidiuretic hormone from the posterior pituitary gland.

A large dose of isoproterenol (0.3 mg/kg) produced peak plasma concentrations of isoproterenol of about 40 ng/ml 10 min after s.c. administration. The total body clearance of isoproterenol was 102 ml kg^{-1} min^{-1}.

Significance to Biomedical Research and Program of the Institute: Although a dose of 0.3 mg/kg is approximately 20,000 times a single dose given i.v. to a human (e.g., 1 µg in a 70 kg man = 14.2 ng/kg), the subcutaneous route of administration and the rapid clearance of isoproterenol in the rat result in surprisingly low plasma levels of isoproterenol. Conolly et al. (Br. J. Pharmac. 46:458,1972) have reported in man a plasma level of 0.39 ng/ml of isoproterenol 1 min after iv injection of 0.063 µg/kg; the peak plasma concentration in our experiments is approximately 100 times this value. An antidiuretic effect accompanied by a fall in GFR and RPF has been reported in human volunteers receiving isoproterenol at intravenous infusion rates of 0.04 to 0.06 µg kg^{-1} min^{-1} (Levi et al., Arch. intern. Med. 136: 25-29, 1976). In this same study, infusion rates of 0.07 to 0.10 µg kg^{-1} min^{-1} could not be tolerated. Thus, humans apparently are far more sensitive to these toxic effects of isoproterenol than are rats.

Proposed Course of Project: A manuscript is now in preparation for publication.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00853-03 LCP

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Modification of Drug Action by Glycofurol

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

PI:	Wilson J. Yasaka	Visiting Fellow	LCP	NHLBI
	Henry Sasame	Chemist	LCP	NHLBI
Other:	M. Negishita	Guest Worker	LCP	NICHHD
	Kenneth Greene	Biol. Lab. Tech.	LCP	NHLBI
	Wilford Saul	Chemist	LCP	NHLBI
	Harriet M. Maling	Chief, Section on Physiology	LCP	NHLBI
	James R. Gillette	Chief, Lab. of Chem. Pharm.	LCP	NHLBI

COOPERATING UNITS (if any)
W. Levin, Hoffmann La Roche, Nutley, New Jersey

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	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)

When glycofurol was administered in multiple doses, it markedly decreased the pharmacological effects of hexobarbital and zoxazolamine, through induction of cytochrome P-450 (Biochem. Pharm. 27:2851,1978). The induction somewhat resembled the induction by phenobarbital and 3-methylcholanthrene of detoxication pathways of xenobiotics. However, a unique property of glycofurol induction was the decreased formation of reactive metabolites of carbon tetrachloride. Therefore, SDS polyacrylamide gel electrophoresis has been used to determine whether a novel form of cytochrome P-450 is induced by glycofurol.

411

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Experiments have been designed to determine the type of induction produced by glycofurol.

Methods Employed: Standard biochemical and electrophoretic techniques were used.

Major Findings: The microsomal induction mediated by glycofurol increased the metabolic rate of 1,2-dimethyl-4-(p-carboxyfenilazo)-5-hydroxybenzene, azo reductase; p-nitro benzoic acid, nitro reductase; quinidine, O-demethylase; p-nitro anisol, O-demethylase; and aminopyrene, N-demethylase. Therefore, glycofurol induction, resemble somewhat either a 3-methylcholanthrene or phenobarbital induction type in these oxidative and reductive reactions. However, when compared the metabolic rate of formation of reactive metabolite of carbon tetrachloride, phenobarbital or 3-methylcholanthrene increase the covalent binding of carbon tetrachloride, in approximately to 50 to 100%. In contrast glycofurol did not increase the covalent binding of carbon tetrachloride. The combination of phenobarbital + glycofurol did not increase the covalent binding of carbon tetrachloride more than phenobarbital alone, but the combination of 3-methylcholanthrene + glycofurol decreased the covalent binding in about 50%.

Examination of the electrophoretic pattern of the solubilized microsomes after staining with Coomassie blue revealed 2 dense bands at 51.0K and 52.5K for control. Microsomes from phenobarbital-induced rats showed two dramatically-increased bands at 49.5K and 53.0K and a band at 51.0K that was slightly increased in the intensity of staining when compared with control microsomes. Microsomes from glycofurol-induced rats showed the same band as microsomes from phenobarbital-induced rats at 49.5K and 51.0K, and also a third dense band at 53.5K which is similar to that seen in microsomes from 3-methylcholanthrene-induced rats; in addition, microsomes from 3-methylcholanthrene-induced rats also showed a band at 56.0K. These findings demonstrate that treatment of rats with glycofurol produces a different pattern of cytochrome P-450. This pattern of haemoproteins maybe responsible for the decreased formation of a reactive metabolite of carbon tetrachloride.

Significance to Biomedical Research and Program of the Institute: These studies suggest that glycofurol pretreatment causes the formation of a novel pattern of cytochrome P-450, which may be responsible in a detoxication mechanism, decreasing the chemical electrophlic adduct that maybe is related to toxicity. The characterization of this hemoprotein could elucidate the mechanism of drug metabolism.

Proposed Course of Project: Experiments will be performed to characterize these haemoproteins, using electroimmunophoresis.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00854-02 LCP

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Mechanism of Hepatic and Renal Toxicity of Phenylbutazone

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

PI:	Wilson J. Yasaka	Visiting Fellow	LCP	NHLBI
	Henry A. Sasame	Chemist	LCP	NHLBI
Other:	Kenneth Greene	Biol. Lab. Tech.	LCP	NHLBI
	Wilford Saul	Chemist	LCP	NHLBI
	Harriet M. Maling	Chief, Sect. on Physiology	LCP	NHLBI
	James R. Gillette	Chief, Lab. of Chem. Pharm.	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:
1.5	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)

Previous studies (Pharmacologist 20:180,1978; Fed. Proc. 38:683,1979) have shown that phenylbutazone (150 mg/kg,i.p.) causes massive hepatic necrosis in rats, especially in those that had been pretreated with both phenobarbital and diethyl maleate. Also the incidence and severity of phenylbutazone-induced hepatotoxicity are correlated with changes in the in vivo covalent binding of chemically reactive metabolites of phenylbutazone after various pretreatments. In vitro studies indicated that covalent binding of phenylbutazone to microsomal protein requires oxygen and NADPH as a cofactor and is inhibited by a CO-O_2 (4:1) atmosphere, and nitrogen. Thus cytochrome P-450 appears to catalyze the formation of these reactive metabolites. The structures of these chemically reactive metabolites remain to be elucidated.

413

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The metabolic fate of phenylbutazone have been studied to determine the toxic pathway responsible for formation of reactive metabolites.

Methods Employed: Biochemical and pharmacokinetic procedures developed by LCP.

Major Findings: In phenobarbital-pretreated rats, phenylbutazone induces liver necrosis and glutathione depletion and the hepatic intrinsic clearance of phenylbutazone is also increased. In contrast, in rats pretreated with 3-methylcholanthrene, phenylbutazone did not induce either liver necrosis or glutathione depletion, and the hepatic intrinsic clearance. The magnitude of *in vivo* covalent binding of phenylbutazone at 6 hours was about the same for both pretreatments. Also *in vitro* covalent binding of phenylbutazone metabolites by rat liver microsomes was about the same for microsomes from rats pretreated with phenobarbital or 3-methylcholanthrene; in other terms, the *in vitro* binding confirms the *in vivo* results.

The formation of these reactive metabolites under phenobarbital or 3-methylcholanthrene are NADPH dependent. However, glutathione inhibited only the covalent binding of microsomes from phenobarbital pretreated rats, with no effect on binding to microsomes from 3-methylcholanthrene pretreated rats. *In vivo*, 3-methylcholanthrene did not deplete glutathione.

These results suggest that covalent binding of phenylbutazone is mediated by the microsomal mixed-function oxidase system, which by the P-450 (phenobarbital) pathway or P-450 (3-methylcholanthrene) pathway can lead to covalent binding. However, P-450 catalyzes the formation of an innocuous chemically reactive metabolite, which is not affected by glutathione; this type of covalent binding thus is not correlated with cytotoxicity. On the other hand, the P-450 pathway induces the formation of a reactive metabolite that binds covalently to macromolecules, and is also inhibited by glutathione; this type of covalent binding is probably related to cytotoxicity.

Significance to Biomedical Research and Program of the Institute: Because phenylbutazone is effective in the treatment of rheumatoid arthritis, it is widely used. Even though the mechanism of hepatotoxicity is still unknown, an understanding of the mechanism of its toxicity may help in the prevention of its side effects.

Proposed Course of Project: The experiments will be performed to characterize the chemically reactive metabolites.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00855-01 LCP

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Estimates of Glomerular Filtration Rate and Renal Plasma Flow in Conscious Rats

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

PI:	Harriet M. Maling	Chief, Section on Physiology	LCP	NHLBI
Other:	Wilford Saul	Chemist	LCP	NHLBI
	Wilson J. Yasaka	Visiting Fellow	LCP	NHLBI
	James R. Gillette	Chief, Lab. of Chem. Pharm.	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:
1.0	0.7	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)

Renal plasma flow and glomerular filtration rate were estimated simultaneously in the conscious rat by the "average total body clearance" of tetraethyl-ammonium (TEA) and inulin, calculated as Dose i.v./AUC, in which AUC represents the area under the plasma disappearance curve of ^{14}C or ^{3}H plasma radioactivity after a single injection into the tail vein of a solution containing both ^{14}C-TEA and ^{3}H-methoxyinulin.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The purpose of this project is to develop a method for estimating renal plasma flow and glomerular filtration rate simultaneously in the conscious rat.

Methods Employed: "Average total body clearance" of tetraethylammonium or inulin is determined as Dose i.v./AUC, in which AUC is the area under the plasma disappearance curve. Plasma levels of radioactivity are measured in blood samples obtained from the retro-orbital sinus at appropriate intervals after the injection in a rat's tail vein of ^{14}C-labeled tetraethylammonium bromide and ^{3}H-methoxyinulin.

Major Findings: Measurement of the clearance of radioactivity from ^{14}C-glycyl labeled p-aminohippuric acid (PAH) was not adequate as an estimate of renal plasma flow (RPF) because PAH was converted into radiolabeled products that interfered with the calculation of AUC. The plasma radioactivity 3 or more hours after injection of ^{14}C-labeled PAH was not PAH, but probably glycine and glycine incorporated into plasma proteins. The clearence of radioactivity from ^{14}C-labeled tetraethylammonium bromide is a valid estimate of RPF.

Significance to Biomedical Research and Program of the Institute: This method of measuring simultaneously in the conscious rat GFR and RFP may be useful in studying the pharmacologic effects of various compounds.

Proposed Course of Project: The method described in this project is being used in other projects.

Publication:

Maling, H.M., Saul, W., Yasaka, W.J. and Gillette, J.R.: Tetraethylammonium as a probe for estimating renal plasma flow in unanesthetized rats. Proc. Soc. Exper. Biol. Med. 161: 88-93, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 00917-04 LCP

PERIOD COVERED
October 1, 1979 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

Studies on the Formation of Reactive Metabolites of Phenacetin and Acetaminophen

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

PI:	Jack A. Hinson	Staff Fellow	LCP	NHLBI
OTHERS:	Lance R. Pohl	Sr. Staff Fellow	LCP	NHLBI
	James R. Gillette	Chief, Lab. of Chemical Pharm.	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme-Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	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)

Evidence has been previously presented that the hepatotoxicity of phenacetin is mediated through the deethylated metabolite acetaminophen followed by activation of acetaminophen to a reactive metabolite. N-acetylimidoquinone was postulated to be the reactive metabolite and was believed to be formed by N-hydroxylation of acetaminophen followed by a spontaneous dehydration. Since the half life of N-hydroxyacetaminophen in aqueous solutions at pH 7.4 was recently shown to be 15 minutes, we examined the possibility that N-hydroxyacetaminophen was a metabolite of acetaminophen and N-hydroxyphenacetin. N-Hydroxyacetaminophen was determined to be a major metabolite of N-hydroxyphenacetin by gas chromatography-mass spectrometry and high pressure liquid chromatography but was apparently not a metabolite of acetaminophen. More covalent binding, however, occurred with acetaminophen as a substrate than with N-hydroxyphenacetin. These data suggest that the chemically reactive metabolites of acetaminophen and phenacetin are not mediated by N-hydroxylation of acetaminophen as previously postulated.

PHS-6040
(Rev. 10-76)

Project No. Z01 HL 00917-04 LCP

Project Description:

Objectives: Previous studies in our Laboratory have shown that the toxicities of phenacetin and acetaminophen are mediated by reactive metabolites formed in the body, but the identities of the reactive metabolites are unknown. The objective of this study is to elucidate possible mechanisms for the formation of reactive metabolites formed from phenacetin and acetaminophen.

Methods Employed: Liver microsomes were isolated from hamster by standard procedures. Gas chromatography-mass spectrometry (GC-MS) was used to identify N-hydroxyacetaminophen as a metabolite. High pressure liquid chromatography (HPLC) was used to quantitate N-hydroxyacetaminophen. All other methods were standard procedure.

Major Findings: N-Hydroxyacetaminophen was found to be a microsomal metabolite of N-hydroxyphenacetin by gas chromatographic assay (HPLC). N-Hydroxyacetaminophen, however, was not found to be a microsomal metabolite of acetaminophen by either GC-MS or HPLC. In the same experiment, more covalent binding occurred using acetaminophen as a substrate, than when N-hydroxyphenacetin was the substrate. These data indicate that N-hydroxylation of acetaminophen is apparently not the mechanism of conversion to its reactive metabolite as has been previously postulated.

Significance to Biomedical Research and Program of the Institute: These data indicate that the mechanism of hepatotoxicity of acetaminophen and phenacetin is not by N-hydroxylation of acetaminophen as previously believed.

Proposed Course of Project: Since the toxicities of acetaminophen and phenacetin are important clinical problems and these toxicities are believed to be mediated by reactive metabolites the problem will be continued.

Publications:

Hinson, J.A., Pohl, L.R. and Gillette, J.R.: N-Hydroxyacetaminophen: A microsomal metabolite of N-hydroxyphenacetin but apparently not of acetaminophen. Life Sciences 24: 2133-2138, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00919-02 LCP

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Mechanism of Bioactivation and Toxicity of Deuterium Labeled Compounds

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

PI:	Lance R. Pohl	Sr. Staff Fellow	LCP	NHLBI
OTHERS:	Jackie L. Martin	Chemist	LCP	NHLBI
	John W. George	Chemist	LCP	NHLBI

COOPERATING UNITS (if any)

I.G. Sipes and A.J. Gondolfi, University of Arizona, Dept. of Anesthesiology, Tucson, Ariz. 85724

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme-Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.2	0.1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Chloroform and bromoform were more hepatotoxic than their corresponding deuterated forms, $CDCl_3$ and $CDBr_3$. These findings demonstrate that the metabolism of the C-H bond of these haloforms is the rate determining step in their mechanism of bioactivation. In contrast, there was no observable difference in the hepatotoxicity produced by iodoform (CHI_3) or halothane ($CF_3CHBrCl$) and their corresponding deuterium labeled derivatives. It thus appears that another biotransformation pathway is the rate determining step in the bioactivation of these compounds into hepatotoxins. The methodology outlined in this report can be applied to studies of the mechanisms of toxicity produced by other halocarbons.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To determine if the metabolism of the C-H bond of bromoform ($CHBr_3$), iodoform (CHI_3), and halothane ($CF_3CHClBr$) is the rate determining step in hepatotoxicity produced by these halocarbons as was found previously with chloroform ($CHCl_3$).

Methods Employed: Deuterium labeled halothane was synthesized by an exchange reaction in sodium deuteroxide (NaOD). $CHCl_3$, $CHBr_3$, CHI_3 and their deuterium labeled derivatives were administered intraperitoneally to male Sprague Dawley rats which were pretreated with phenobarbital. Halothane and deuterium labeled halothane were administered to the rats by inhalation for 2 hours. After 24 hours, the degree of hepatotoxicity was measured both histologically and enzymatically.

Major Findings: As found previously, $CDCl_3$ was approximately 2 times less hepatotoxic than $CHCl_3$. Similarly, $CDBr_3$ was less hepatotoxic than $CHBr_3$. In contrast, there was no discernible difference in the hepatotoxicity of $CF_3CDBrCl$ and $CF_3CHBrCl$, or CDI_3 and CHI_3. The observations that $CDCl_3$ and $CDBr_3$ are less hepatotoxic than $CHCl_3$ and $CHBr_3$ respectively indicates that the metabolism of the C-H bond is the rate determining step in the hepatotoxicity produced by these compounds. The oxidative dehalogenation of these haloforms to the reactive and toxic carbonyl halides (COX_2) is a likely pathway responsible for these results, because $CHCl_3$ and $CHBr_3$ were previously found to be oxidatively dehalogenated in liver microsomes to $COCl_2$ and $COBr_2$ respectively. Moreover, the deuterium labeled derivatives were metabolized to the COX_2 more slowly than the protio-compounds.

In the case of iodoform and halothane, there was no apparent difference in the hepatotoxicity of the protio and deuterated derivatives. This finding indicates that the metabolism of the C-H bond of iodoform and halothane, unlike the $CHCl_3$ and $CHBr_3$, is not the rate determining step in their hepatotoxicity. Therefore, another pathway of metabolism appears to be involved in the formation of the hepatotoxic metabolite or metabolites of these compounds. The reductive cleavage of the C-I bond of CHI_3 or the C-Cl or C-Br bonds of halothane are potential pathways of activation of these compounds.

Significance to Biomedical Research and the Program of the Institute: The results of these experiments confirm the general use of deuterium labeled compounds for the elucidation of pathways of bioactivation of various halocarbon drugs and environmental chemicals.

Proposed Course of Project: We plan to continue our studies with halothane in order to elucidate how this halocarbon is bioactivated into a hepatotoxin. In addition, we also intend to use the same methodology outlined in this report to determine the mechanism of toxicity of other halocarbons, such as the general anesthetic enflurane.

Publications:

Pohl, L.R. and Krishna, G.: Deuterium isotope effect in bioactivation and hepatotoxicity of chloroform. Life Sci. 23: 1067-1072, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00921-01 LCP

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

High Pressure Liquid Chromatographic Assay of Hydroxamic Acids

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

PI:	Jack A. Hinson	Staff Fellow	LCP	NHLBI
	Lance R. Pohl	Sr. Staff Fellow	LCP	NHLBI
Other:	James R. Gillette	Chief, Lab. of Chemical Pharm.	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:
0.1	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)

Hydroxamic acids (N-hydroxyarylamides) have been implicated in various toxicities including the carcinogenicity produced by 2-acetylaminofluorene and other polycyclic N-acetylarylamides and the cell necrosis produced acetaminophen and phenacetin. These compounds, however, are very difficult to assay by conventional methods. A specific high pressure liquid chromatographic assay for hydroxamic acids has thus been developed. In this assay ferric chloride is included in the elution solvent and the hydroxamic acids are isolated and detected as ferric chelates at 546 nm. The assay is very sensitive with a number of hydroxamic acids including those of 2-acetylaminofluorene, phenacetin, acetaminophen and acetanilide. There was only minimal interference from isomeric phenols.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: We have found that hydroxamic acids are difficult to assay by conventional techniques. For example, on silica gel thin layer chromatography they adsorb to the silica gel and appear as smears on the chromatographic plate. In high pressure liquid chromatographs they do not elute from reverse phase column (C_{18} μBondapak column) even with methanol or acetonitrile. We, therefore, developed a sensitive method to quantitate these compounds.

Methods Employed: The hydroxamic acids were synthesized by previously described techniques. Ferric chloride (1 mM) was added to the methanol:water elution solvent and a C_{18} μBondapak column was used. All other techniques were standard procedure.

Major Findings: A specific high pressure liquid chromatographic assay for hydroxamic acids of 2-acetylaminofluorene, phenacetin, acetaminophen and acetanilide has been developed by including ferric chloride (1 mM) in the elution solvent (methanol:water). In this assay the ferric ion combines with the hydroxamic acid to form a chealte that is eluted from the column and detected at 546 nm. The phenols that are formed during the decomposition of the hydroxamic acids do not appear to interfere with the method. This liquid chroamtographic assay gives a dependable procedure for the analysis of hydroxamic acids which are believed to be toxic metabolites of N-acetylarylamides. The lower limit of sensitivity was approximately 25 ngm.

Significance to Biomedical Research and Program of the Institute: This assay is a general method for quantitation of hydroxamic acids which are believed to be important in various toxicities of N-acetylarylamides.

Proposed Course of Project: The assay will be used to quantitate various hydroxamic acids.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00922-01 LCP

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

3-Hydroxyacetaminophen: An acetaminophen metabolite

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

PI:	Jack A. Hinson	Staff Fellow	LCP	NHLBI
	Lance R. Pohl	Sr. Staff Fellow	LCP	NHLBI
Other:	Terrence J. Monks	Visiting Fellow	LCP	NHLBI
	James R. Gillette	Chief, Lab. of Chem. Pharm.	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme-Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	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)

3-Hydroxyacetaminophen, which has been identified by gas-chromatography mass spectrometry and liquid chromatography as a liver microsomal metabolite of acetaminophen, has been quantitated by a liquid chromatographic assay and a catechol O-methyl transferase plus [^{3}H-methyl] S-adenosyl methionine assay. Its formation is apparently unrelated to the formation of the reactive toxic metabolite of acetaminophen since ascorbic acid and glutathione, both of which block covalent binding of the reactive metabolite of acetaminophen to protein, had little affect on its formation. Moreover, in microsomal incubation mixtures containing ^{3}H-acetaminophen plus NADPH, its formation apparently does not lead to appreciable amounts of covalent binding since neither superoxide dismutase nor catechol O-methyl transferase plus S-adenosyl methionine blocked covalent binding of the radiolabeled acetaminophen to protein.

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Project Description:

Objectives: We have been interested in the mechanism of toxicity of acetaminophen and related compounds. Since the toxicity of acetaminophen has been previously shown to be mediated by a microsomal metabolite which will covalently bind to protein we have looked for metabolites which may be formed from the reactive metabolite.

Methods Employed: Hamster liver microsomes were isolated and were incubated with ^{3}H-acetaminophen plus NADPH. An ethyl acetate extract of the incubation evaporated under a stream of nitrogen, silated, and analyzed by gas chromatography-mass spectrometry. 3-Hydroxyacetaminophen standard was synthesized by reduction of 4-nitrocatechol followed by acetylation of the aminocatechol. The formation of 3-hydroxyacetaminophen was analyzed by liquid chromatography of ethyl acetate extracts of the microsomal incubation mixtures and by a catechol O-methyl transferase plus [^{3}H-methyl]-S-adenosyl methionine assay. All other methods were standard procedures.

Major Findings: 3-Hydroxyacetaminophen was identified as a microsomal metabolite of acetaminophen by gas chromatography-mass spectrometry. In the presence of an $^{18}O_2$ atmosphere there was approximately a 100% incorporation of molecular oxygen-18 into the metabolite indicating the oxygen was derived atmospheric oxygen and not from water.

Catechols have been previously shown to be oxidized in microsomal incubation mixtures to species which covalently bind to protein. Since a reactive metabolite of acetaminophen which covalently binds to protein has been implicated in acetaminophen induced hepatotoxicity, its relationship to 3-hydroxyacetaminophen was investigated. Neither superoxide dismutase nor catechol O-methyl transferase plus S-adenosyl methionine had any effect on binding of the reactive metabolite of acetaminophen to protein. Although both glutathione and ascorbic acid inhibit covalent binding of the reactive metabolite of acetaminophen, to protein neither had any effect on the formation of 3-hydroxyacetaminophen as measured by a liquid chromatographic assay or by a catechol O-methyl transferase plus [^{3}H-methyl] S-adenosyl-methionine assay. Thus the formation of 3-hydroxyacetaminophen and the reactive metabolite of acetaminophen are apparently unrelated.

Significance to Biomedical Research and Program of the Institute: These data indicate that an epoxide of acetaminophen which rearranges to 3-hydroxyacetaminophen is apparently not the reactive metabolite of acetaminophen.

Proposed Course of Project: The toxicity of 3-hydroxyacetaminophen will be investigated.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00923-01 LCP

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Phosgene: An in vivo metabolite of chloroform

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

PI:	Lance R. Pohl	Sr. Staff Fellow	LCP	NHLBI
Other:	Jackie L. Martin	Chemist	LCP	NHLBI
	John W. George	Chemist	LCP	NHLBI
	Gopal Krishna	Chief, Section on Drug-Tissue Inter.	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:
0.5	0.2	0.3

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Phosgene ($COCl_2$) was identified as a metabolite of $CHCl_3$ in rat liver in vivo by trapping it with cysteine as 2-oxothiazolidine-4-carboxylic acid. When the experiment was repeated with a 1:1 mixture of $CDCl_3$ and $[^{13}C]CHCl_3$, approximately twice as much $COCl_2$ was trapped from $[^{13}C]CHCl_3$ as from $CDCl_3$. This finding indicates that a deuterium isotope effect is involved in the formation of $COCl_2$. Moreover, it appears that $COCl_2$ is responsible, at least in part, for the hepatotoxicity produced by $CHCl_3$ because $CDCl_3$ is less hepatotoxic than $CHCl_3$. We believe that the use of stable isotopes and mass spectroscopy outlined in this report can be used to determine the toxic metabolites of other halocarbon compounds.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To determine if phosgene ($COCl_2$) is a metabolite of $CHCl_3$ *in vivo* and whether it could be responsible, at least in part, for the hepatotoxicity produced by $CHCl_3$.

Methods Employed: In a typical experiment, phenobarbital pretreated rats were treated with cysteine (1 gm/kg, i.p.) followed by $CHCl_3$ (4.98 mmole/kg, i.p.) 30 min later. After 1 hr, livers were removed and analyzed for $COCl_2$ as the cysteine conjugate, 2-oxothiazolidine-4-carboxylic acid by GCMS.

Major Findings: A fraction with the same retention time and MS as the synthetic standard was detected in an extract of liver. The identity of fraction as trapped $COCl_2$ was confirmed by repeating the study with $[^{13}C]CHCl_3$. An additional investigation was performed with a 1:1 mixture of $CDCl_3$ and $[^{13}C]CHCl_3$. Approximately twice as much $COCl_2$ was trapped from $[^{13}C]CHCl_3$ as from $CDCl_3$, a finding which is consistent with studies using rat liver microsomes.

Significance to Biomedical Research and Program of the Institute: We believe that the use of stable isotopes and mass spectroscopy outlined in this report has general application and can be used to determine the toxic metabolites of other halocarbon compounds such as halothane and enflurane.

The larger amount of $COCl_2$ produced from $CHCl_3$ than from $CDCl_3$ *in vivo* indicates that the breakage of the C-H bond of $CHCl_3$ is the rate determining step in the formation of this metabolite. Moreover, it appears that $COCl_2$ is responsible at least in part, for the hepatotoxicity produced by $CHCl_3$ because $CDCl_3$ is less hepatotoxic than $CHCl_3$.

Proposed Course of Project: We intend to study further the metabolism of $CHCl_3$ *in vitro* and *in vivo* in order to determine if metabolites other than $COCl_2$ are involved in its toxicity. We feel these studies will serve as a model for the elucidation of toxic pathways of metabolism of structurally more complicated halocarbons. Information from such metabolic studies will ultimately permit the design of safer drugs.

Publications:

Pohl, L.R., George, J.W., Martin, J.L. and Krishna, G.: Deuterium isotope effect in *in vivo* bioactivation of chloroform to phosgene. Biochem. Pharmacol. 28: 561-563 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00924-01 LCP

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Mechanism of Halothane Bioactivation

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

PI:	Lance R. Pohl	Sr. Staff Fellow	LCP	NHLBI
Other:	John W. George	Chemist	LCP	NHLBI
	Jackie L. Martin	Chemist	LCP	NHLBI

COOPERATING UNITS (if any)
I.G. Sipes and A.J. Gondolfi, University of Arizona, Dept. of Anesthesiology, Tucson, Ariz. 85724

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme-Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.2	0.1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The mechanism of bioactivation of halothane by liver microsomes of rats was investigated by measuring the covalent binding of [^{14}C] and [^{3}H] halothane to microsomal protein and lipid under atmospheres of O_2 and N_2. Under O_2, only the [^{14}C] label of halothane bound appreciably to protein and lipid. In contrast, both labels bound covalently to protein and lipid, when the incubations were performed under N_2. These results indicate that at least two mechanisms are involved in the bioactivation of halothane by liver microsomes. In the presence of O_2, halothane is likely activated by an oxidative dehalogenation mechanism, as was found previously for $CHCl_3$. The activation under low oxygen tension may involve the dehalogenation of halothane to a radical or carbene metabolite.

428

Project No. Z01 HL 00924-01 LCP

Project Description:

Objectives: We have been able to determine more clearly the mechanism of bioactivation of $CHCl_3$ by liver microsomes with [^{14}C] and [^{3}H] labeled derivatives. The approaches used in these studies have now been extended to investigate the mechanism of metabolic activation of the halogenated anaesthetic halothane ($CF_3CHBrCl$).

Methods Employed: [^{3}H] labeled halothane was synthesized by an exchange reaction with an alkaline solution of tritiated water. [^{14}C] and [^{3}H] labeled halothane were incubated with liver microsomes of rat under atmospheres of O_2 or N_2 and the covalent binding to microsomal protein and lipid were measured.

Major Findings: When the incubations were conducted in an atmosphere of O_2 [^{14}C] halothane was bioactivated and bound covalently to protein (334 picomoles/mg protein) and lipid (1260 pmoles/μmole lipid), but much smaller quantities of [^{3}H] halothane were bound to protein (<20 pmoles/mg protein) and lipid (168 pmoles/μmole lipid). Incubation of microsomes under N_2 resulted in a two-fold increase in binding of [^{14}C] halothane to protein (686 pmole/mg protein) and a three-fold increase to lipid (3860 pmole/μmole lipid) over that observed with O_2. Interestingly, the anaerobic incubation of [^{3}H] halothane resulted in approximately a twelve-fold increase in the binding to both microsomal protein (232 pmole/mg protein) and lipid (2566 pmole/μmole lipid).

Significance to Biomedical Research and the Program of the Institute: These studies indicate that halothane is bioactivated under aerobic and anaerobic conditions by at least two different mechanisms. In the presence of O_2, the C-H bond of halothane appears to be cleaved during metabolic activation because very little [^{3}H] halothane binds under these conditions. A mechanism which explains these results is the oxidation of halothane ($CF_3CHBrCl$) to $CF_3COHBrCl$, followed by spontaneous dehydrobromination to trifluoroacetyl chloride (CF_3COCl). This reactive electrophile would be expected to trifluoroacylate protein and lipids. A similar oxidative dehalogenation mechanism of activation has been shown to occur with $CHCl_3$.

Since nearly as much [^{3}H] label binds to microsomal protein and lipid as the [^{14}C] label when the incubations are performed anaerobically, another mechanism of bioactivation is involved under these conditions. One potential pathway of activation would involve reductive cleavage of halothane to CF_3CHCl radical. Another potential reactive metabolite which would contain both [^{14}C] and [^{3}H] labels is CF_3CH carbene.

Proposed Course of Project: We intend to elucidate the mechanisms of metabolic activation of halothane. With this knowledge we feel that we can better understand how halothane causes liver necrosis. Our ultimate goal is to design a safe general anaesthetic that is eliminated from the body by nontoxic routes of biotransformation.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00925-01 LCP

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Synthesis of Tritium Labeled Chloramphenicol and Analogues of It

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

PI:	Lance R. Pohl	Sr. Staff Fellow	LCP	NHLBI
Other:	Jackie L. Martin	Chemist	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Enzyme-Drug Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The 1-hydroxy epimers of chloramphenicol and thiamphenicol formed from the reduction of the respective 1-oxo derivatives with $[^3H]NaBH_4$ have been separated preparatively by high pressure liquid chromatography on a μBondapak C_{18} column. This separation procedure permits the facile and rapid preparation of the [1-^{3}H] labeled derivatives of chloramphenicol and its analogues.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To develop a facile and rapid method for the synthesis of chloramphenicol and its analogues of high specific radioactivity.

Methods Employed: The 1-oxo ketone derivatives of chloramphenicol (CAP) and thiamphenicol (TAP) were prepared by oxidation with N-bromosuccinimide. The ketone products were then reduced with [^{3}H]$NaBH_4$ to a mixture of R,R, and S,R epimers. These isomers were separated and purified by preparative HPLC using a Waters Associates chromatograph which was equipped with a μBondapak C_{18} Column (7.8 mm x 30 cm). The mobile phase consisted of mixtures of methanol and water which were pumped through the column at 3 ml/min.

Major Findings: The [^{3}H] labeled isomers of CAP and TAP were prepared in an overall yield of 59% for CAP and its epimer and 56% for TAP and its epimer.

Significance to Biomedical Research and Program of the Institute: Until now, the synthesis of [^{3}H] labeled CAP of high specific activity has involved several synthetic steps, which take a great deal of time. The present method involves two chemical reactions which are rapid and easy to perform on a small scale. The epimers, which are produced after reduction with $NaBH_4$, are rapidly separated and purified by HPLC. This procedure appears to represent a general method for the synthesis of [^{3}H] labeled analogues of CAP, such as TAP. These radiolabeled compounds should be useful for studies on the pharmacology, biotransformation, and mechanism of action of CAP and its analogues. In addition, the labeled epimers of these compounds may be used to probe the influence of stereochemistry in these studies.

Proposed Course of Project: The method will be used to prepare other [^{3}H] labeled derivatives of CAP.

Publications:

Martin, J.L., Taburet, A.M. and Pohl, L.R.: The use of high pressure liquid chromatography in the preparation of tritium labeled chloramphenicol and its analogues. Anal. Biochem., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00926-01 LCP

PERIOD COVERED

October 1, 1979 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

A Simple and Sensitive Method for Estimation of Chloramphenicol and Thiamphenicol

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

P.I.:	Gopal Krishna	Chief, Sect. on Drug-Tissue Inter.	LCP	NHLBI
OTHERS:	Christine McDaniels	Biological Tech.	LCP	NHLBI
	Nancy Kim	Chemist	LCP	NHLBI
	Tom Hundley	Biological Tech.	LCP	NHLBI

COOPERATING UNITS (if any)

B.G. Reddy, FDA and DRS

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)

A simple and sensitive method has been developed for the routine assay of chloramphenicol and thiamphenicol utilizing an enzyme isolated from E. Coli. This method has been applied for the measurement of thiamphenicol and chloramphenicol in plasma. After oral administration of thiamphenicol (100 mg/kg) only levels of 5-10 µg/ml of the drug was obtained in plasma. After intramuscular administration of chloramphenicol succinate in monkey drug disappeared very rapidly with a half life of 1-2 hrs., indicating that chloramphenicol should be administered more frequently in monkeys in order to maintain adequate antibiotic level.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: In order to understand the mechanism involved in chloramphenicol induced aplastic anemia it became necessary to monitor the drug levels in plasma in calves during the treatment with the drugs. Even though there are a number of methods available to monitor these drug levels none of them were amenable for routine assay for a large number of samples which were involved in this study. In order to overcome this difficulty we have developed a simple assay of both thiamphenicol and chloramphenicol utilizing ^{14}C-acetyl, CoA, and an enzyme which specifically and quantitatively converts these compounds to ^{14}C-diacetylated derivatives. This method does not involve prior extraction of the compound from plasma and utilizes only 10 µl of plasma for the assay.

Methods Employed: Chloramphenicol acetyltransferase (CAT) was isolated from a strain of E coli carrying a plasmid for this enzyme. The enzyme isolation did not involve any complicated process except sonication to achieve solubilization of membrane bound enzyme. The enzyme is stable and may be stored at -20°C for more than a year without any loss of activity. The assay for thiamphenicol and chloramphenicol is plasma involved incubation of 10 µl of sample with CAT (2-3 mU) with ^{14}C-acetyl CoA (100 nmoles-40nCi) in 100 mM Tris HCl pH 7.8 (final volume 100 µl). At the end of 60 min incubation at 37°C, ^{14}C-diacetylated derivative of chloramphenicol was extracted with 2 ml of ethylacetate. Aliquots (1 ml) were taken for radioactivity determinations.

Major Findings: The assay is quite simple and it is possible to routinely assay hundreds of samples per working day. Moreover the assay is quite sensitive and it is possible to measure less than 10 ng of both chloramphenicol and thiamphenicol. This assay has been utilized for the monitoring plasma levels of thiamphenicol in calves and chloramphenicol in monkeys. After administration of 100 mg/kg thiamphenicol orally to calves, the plasma level of the drug rose gradually during the first 4 hrs reaching a peak level of 3 µg/ml which was maintained up to 8 hrs which gradually decline - 0.1 µg/ml at 24 hrs. Administration of the drug on a daily basis at 100 mg/kg/day increased the plateau plasma level to 5 µg/ml on 1st day and 10 µg/ml on 6th day. This level was not changed greatly by continued administration of the drug daily during next 4 days.

Intramuscular administration of chloramphenicol succinate into monkeys at 25, 50 and 75 mg/kg produced peak levels of 5, 8, and 10 µg/ml in 1 hr which declined with an average half life of 1.1, 1.5 and 1.65 hrs respectively. It thus appears that chloramphenicol may have to be administered quite frequently to maintain an adequate antibiotic level in monkeys.

Significance to Biomedical Research and Program of the Institute: With the advent of a development of a simple method for monitoring chloramphenicol and thiamphenicol plasma levels should greatly aid in the understanding of toxicities produced by these compounds in calves. Moreover, it should be

possible to use this method for routine monitoring plasma levels in children since the amount of plasma sample required for this assay is quite small.

Proposed Course of Project: We plan to use this method for monitoring chloramphenicol and thiamphenicol levels both in animals and in vitro experiments utilizing isolated cells.

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, 1978, through September 30, 1979

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: isolation and analysis of biologically important compounds using gas and high pressure liquid chromatography, structural analysis using mass spectrometry, nuclear magnetic resonance, X-ray single crystal methods, and finally computer organization and analysis of scientific data.

Efforts have continued in the development of liquid chromatography. We (T. Jaouni, H. Fales) have developed an analytical system for verapamil in plasma that is reliable at the nanogram level. Key to success of the method is a Schoeffel fluorimetric detector. The method is currently being employed by the Cardiology Branch (M. Leon). In other applications, we (H. Lloyd) have been able to separate the suspected carcinogens sesamin and sesamolin as well as the anacardic acids that affect platelet aggregation and prostacyclins.

In mass spectrometry, the laboratory's venerable MS-9 spectrometer has finally been fully reconditioned and is working satisfactorily although it still requires a great deal of attention. The newer LKB-2091, on evaluation for 2 years, has been purchased and provides excellent spectra especially with capillary columns. A chemical ionization attachment is being purchased for it to enhance molecular ion identifications. The ^{252}Cf plasma desorption instrumentation discussed last year is still in the procurement mill and should arrive in late fall.

As mentioned last year, the advantages of high magnetic fields in the nmr of biological systems is increasingly clear. In collaboration with DCRT (J. Ferretti) and NIMH (J. Costa) we have ordered a 360 MHz system (Nicolet Inst. Co.) that will enable us to be active in this area next year. This acquisition comes at an ideal moment because the Varian XL-100 spectrometer has had increasing problems stemming from the fact that service for the Digilab computer system is no longer available. Meanwhile, the existing JEOL ^{13}C nmr system continues to provide excellent data on a variety of problems. In particular, apolipoprotein AII, treated with ^{13}C iodoacetic acid has allowed observation of a single enhanced resonance line as the protein undergoes modification by guanidine treatment.

In X-ray crystallography, our diffractometer has been updated to allow Fortran programming. With this system, Dr. Silverton will write programs to facilitate assignment of absolute configuration. Many new structures have been completed this year as their molecular weight increases aboye 1000 daltons. R-factors well below 3.7% (bond length e.s.d.s $\pm$ 0.004A) have been achieved. Interesting structures studied include a synthetic cyclic polypeptide, colchiceine, 7-ketodihydromorphinan, a carbomethoxy-carboline, rohitukine and dibenzoylstilbene.

The NIH/EPA Chemical Information System, heavily supported in recent years by NHLBI has now 26 different components (spectrometric and bibliographic data). Several utilities have been added to allow easier use of the system, including a CIS News system, cost analysis program, user status check program, and an off-line listing generation program. It is now possible for a user to transfer from one CIS component to another without logging on and off. Because of its heavy load on DIR at NHLBI, a decision has been made to transfer the system to the Division for Research Resources in the next fiscal year. Dr. Milne will remain, at least temporarily, the project's supervisor until its role within DRR can be completely defined.

Novel compounds whose structures have been elucidated in our laboratory this year include: a new metabolite in the GABA pathway, 5-hydroxy-2-pyrrolidone, a new kaurene-triol from Helichrysum dendroideum, the first example of a naturally occurring S-methylthiocarbonate, paederoside, from Paederia scandens, several secretions from various insects (ants, termites, pentamoidae, beetles, bees) the first naturally occurring polyester from a bee (Colletes), poly ω-hydroxyoctadecanoic and ω-hydroxyeicosanoic acid, and a series of novel long, short, short triglycerides from Anthophora solitary bees,

ANNUAL REPORT OF THE
LABORATORY OF CHEMISTRY
SECTION ON PHYSIOLOGICAL CHEMISTRY
NATIONAL HEART, LUNG AND BLOOD INSTITUTE

October 1, 1978 through September 30, 1979

Kallikrein-Kinin System: New polypeptide inhibitors of Hageman factor, factor X, thrombin and kallikreins from plasma and urine have been isolated from plant materials. Some of the inhibitors have a unique spectrum of activity and specificity against serine proteinases. K_m and k_{cat} values were determined for human Hageman factor fragments with plasmas prokallikrein and six synthetic substrates. A monospecific antiserum to human Hageman factor was obtained in sheep. SDS-polyacrylamide gel electrophoresis of the four main electrofocusing forms of human plasma prokallikrein gave a band a M_r=84,000 for each. The pI 9.3 form also had an equally strong band at M_r=80,000, while the pI 9.1 and 8.8 forms had progressively weaker bands at this M_r. HF_f treatment gave the same pattern. Sheep antiserums to the pI 8.8 prokallikrein and to the kallikrein derived from it were monospecific except for some anti-IgG in the antiserum to prokallikrein. Human plasma kininogens, prepared by immunoaffinity chromatography in the presence of Polybrene and several serine protease inhibitors, gave a DEAE-cellulose chromatographic profile very similar to that found previously for kininogens purified in the presence of Polybrene only. A radioimmunoassay (RIA) for human high molecular weight kininogen (HMWK) has been improved and more firmly established. Extensive cross-reactivity studies have been performed. The assay has been applied to 17 normal plasmas and showed excellent correlation with bioassay. Gel filtration of normal plasma showed no antigenic fragments. Seven plasmas reportedly deficient in HMWK also have been studied using the RIA. Antigen has been detected in urine. Pepsin releases kinin only from kininogen molecules which contain the kinin moiety at the carboxyl end. Radioimmunoassays are under development for human urinary kallikrein, urokininogen, and total plasma kininogen. High molecular weight kininogen is elevated in plasma of individuals on a high salt intake. A patient with hereditary angioedemia had only half the normal level of high molecular weight kininogen.

Determinants of Hormone Action: Arginine vasopressin (AVP) added to the serosal bath of an isolated toad urinary bladder preparation in addition to increasing water flow caused a dose-dependent release of proteinase(s) into the mucosal but not the serosal fluid. Low molecular weight serine proteinase inhibitors added to either the serosal or mucosal fluid significantly inhibited the AVP-induced water flow and the elaboration of the proteinase(s). Trypsin added to the serosal (but not the mucosal) surface induced an AVP-like water flow without increasing the proteinase activity in the mucosal fluid. cAMP also induced water flow without a detectable increase in proteinase activity in the mucosal fluid. One inhibitor tested so far, which effectivley inhibited AVP and trypsin, did not alter the cAMP-induced water flow.

Peptide Biochemistry: Using novel substrates synthesized in our laboratory, a new enzyme was discovered which splits the dipeptide prolylarginine from hippurylprolylarginine and prolylalanine from glycylprolalanine. The enzyme has been tentatively named X-prolyldipeptidyl carboxypaptidase.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01002-06 CH

PERIOD COVERED

October 1, 1978, to September 30, 1979

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
OTHER	J. Osborne	Chemist	MD IR NHLBI
	J. Ferretti		CR PSL

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemistry

SECTION

Structural Nuclear Magnetic Resonance Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Carbon-13 Nuclear Magnetic Resonance spectroscopy is used to investigate intra-molecular and inter-molecular interactions of Apo-Lipoproteins. Chemical shifts and half-band with changes are examined in the presence and absence of guanidine-hydrochloride. Spin-lattice relaxation times (T_1) determinations are being performed with complexes of AI and AII.

PHS-6040
(Rev. 10-76)

The investigation of the apo-lipoprotein AII by C-13 nmr is in the early stages of data collection. The natural abundance C-13 spectra gave little resolution of individual carbon atoms at the field strength employed. Enrichment by reduction and alkylation of disulfide bonds with iodo (C-13) acetic acid allowed observation of a single enhanced resonance.

Titration of the A-II with guanidine-hydrochloride in hopes of destroying interactions between monomer chains showed no shift or half-bandwidth chances in the resonance. Because this treatment has shown changes in spectrophotometric and circular dichoism measurements, it is currently believed that the enriched carbon is not greatly involved at the sight of interactions. Enrichment at other sights are planned to further clarify this possibility.

Solutions of mixed AII and AI apo-lipoproteins gave some chemical shift variations from the AII alone. Spin lattice relaxation measurements of these solutions are in progress.

Publications

1. Ortner, M. J., Sir, Robert H. and Sokoloski, E. A. A nuclear magnetic resonance study of compound 48/80. Mol. Pharm., 15 179-188 (1979).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01003-08 CH

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Structure of Natural Products Using Instrumental Methods

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

PI:	H. M. Fales	Chief, Lab. of Chem.	CH	NHLBI
OTHER:	T. Jaouni	Chemist	CH	NHLBI

COOPERATING UNITS (if any)
none

LAB/BRANCH
Laboratory of Chemistry

SECTION
Chemical Structure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A method has been developed for the assay of verapamil in plasma at nanogram/ml levels.

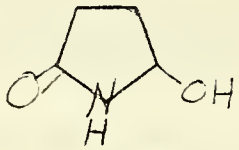
A new intermediate in GABA metabolism, 5-hydroxy 2-pyrrolidone has been identified and synthesized.

A series of insect products, including the first natural polyester and a series of unusual triglycerides have been identified and characterized.

A technique for detecting dehydrogenation in GC-MS systems has been developed.

PHS-6040
(Rev. 10-76)

With D. Lundgren (NIAMDD), the structure of a compound elaborated during the biosynthesis of 2-pyrrolidone has been elucidated as 5-hydroxy2-pyrrolidone (1).



This compound had been prepared earlier from glutamine by Meister et al., but its biological role was unsuspected. We have studied its properties and synthesized a radioactive form for further study.

An analytical method for the determination of verapamil in plasma has been developed using fluorometric detection and high pressure liquid chromatography. The technique depends on two internal standards and is capable of good accuracy and linearity in the range of 1-100 ng of plasma, i.e., the amounts typically found in 1 ml of plasma during therapy (M. Leon Cardiology Branch, NHLBI).

With S. Batra (USDA, Beltsville) the constituents of the cell linings of several species of solitary bees have been investigated. In one case, (Colletes) the lining consists of a polymer of ω-hydroxyoctadecanoic and ω-hydroxyeicosonoic acids, perhaps the first example of a naturally occurring polyester. In another case (Anthophora) the lining consists of a diglyceride, (excreted as a triglyceride) from the Dufour's gland of the female bee. The triglycerides are an unusual series of long, short, short triglycerides similar to those found in a the bottle-nose dolphin. An enzyme, perhaps in the saliva of the bee, converts the triglyceride to diglyceride which serves first for cell construction and ultimately for food for the larvae. Work is continuing to determine relevance of this system, if any, to the salivary lipases under investigation by R. Scow, NIAMDD.

With M. Blum (Dept. of Entomology, Univ. of Georgia) the identity of a series of pheromones related to the killer bee has been established. Contrary to current opinion, the pattern of compounds elaborated by the sting apparatus (octyl & decyl esters and alcohols) is substantially the same as ordinary honeybees. The difference in behavior is thus presumably genetic in character.

A system using 2-butyl-5-amylpyrrolidine has been found to be a sensitive indicator of dehydrogenation and sample destruction in gas chromatographic-mass spectrometer systems and is recommended as a general test substance to disclose this ubiquitous problem.

Project No. Z01 H1 01003-08 CH

Publications:

1. Blum, M. S., Wallace, J. B., Duffield, R. M., Brand, J. M., Fales, H. M. and Sokoloski, E. A. Chrysomelidial in the defensive secretion of the leaf beetle. Gastrophysa cyanea Melsheimer. J. Chem. Ecol. 4: 47-53, 1978.

2. Aldrich, J. R., Blum, M. S., Hefetz, A., Fales, H. M., Lloyd, H. A., and Roller, P. Proteins in a nonvenomous defensive secretion: biosynthetic significance. Science 201: 452-454, 1978.

3. Aldrich, J. R., Blum, M. S., Lloyd, H. A., and Fales, H. M. Pentatomid natural products. Chemistry and morphology of the III-IV dorsal abdominal glands of adults. J. Chem. Ecol. 4: 161-172, 1978.

4. Jones, T. H., Blum, M. S., and Fales, H. M. Synthesis of unsymmetrical 2,5-di-n-alkylpyrrolidines: 2-hexyl-5-pentylprrolidine from the thief ants Solenopsis molesta, S. texanas and its homologues. Tetrahedron Lett 12: 1031-1034, 1979.

5. Blum, M. S., Fales, H. M., Tucker, K. W., and Collins, A. M. Chemistry of the sting apparatus of the worker honeybee. J. of Apicultural Res. 17: 218-221, 1978.

6. Lloyd, H. A., Evans, S. L., and Fales , H. M. Terpene alcohols of Helichrysum dendroideum II. Lloydia 21: 494-496, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01004-08 CH

PERIOD COVERED

October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

Characterization of natural products

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

PI: H. A. Lloyd Research Chemist CH NHLBI

COOPERATING UNITS (if any)

none

LAB/BRANCH

Laboratory of Chemistry

SECTION

Chemical Structure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

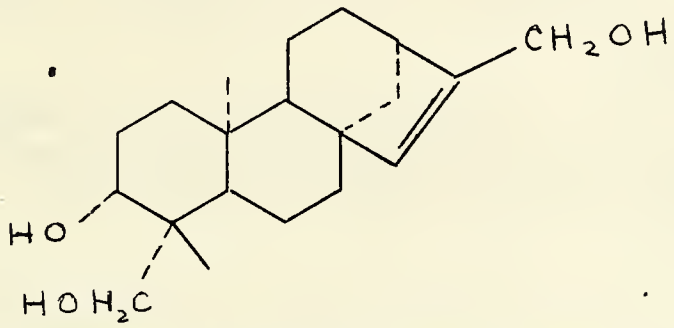
The purpose of this work is to determine the structures of new biologically active compounds of plant or animal origins. These products are studied by physical techniques such as nmr, mass spectrometry and chemical methods such as synthesis and degradation.

444

Project No. Z01 HL 01004-08 CH

A. Plant Products

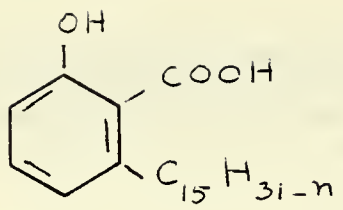
1. Work on the diterpene alcohols of Helichrysum dendroideum has continued. The structures of three mono alcohols were established and a new trialcohol was isolated. Study of its nmr and of its hydrogenation products suggested a kaurene type skelton and an allylic alcohol group.



2. (In collaboration with Dr. G. Kapadia, Howard University). The iridoid glucoside, paederoside, isolated from Paederia scandens, has been shown to be the first example of a naturally-occurring S-methyl thiocarbonate ($R-CH_2-OOC-S-CH_3$). Previous researchers had assigned a thioacetate structure ($R-CH_2-S-COCH_3$) to this compound. The new structure was established with the aid of electron impact and chemical ionization mass spectrometry, nmr and by synthesis of model compounds.

3. The potential carcinogens in sesame and olive oil were investigated (with Dr. G. Kapadia, Howard U.) Sesame oil contains two carcinogens, sesamin and sesamolin, which cannot be separated entirely by crystallization, thin-lay or column chromatography or by chemical means. An excellent separation was achieved by high pressure liquid chromatography.

4. Long-chain salicylic acids ("anacardic acid") from cashew nut shells, Anacardium occidentale (in collaboration with Dr. G. Krishna, CP, NHLBI). These compounds are interesting for their action on platelet aggregation and prostacyclin inhibition. The crude extract is a molluscicide and has been used in South America to kill snails carrying Schistosomia parasites. The "anacardic acid" was separated from the phenolic fraction and resolved in its saturated, monoene, diene and triene constituents by high pressure liquid chromatography. The various acids were further characterized by nmr, derivatization and mass spectrometry.



$n = 0, 2, 4, 6$

B. Insect Natural Products

The secretion of a number of ants (Myrmecocystus), african termites (mono and sesquiterpenes), beetles (isocoumarins), stinkbugs (aldehydes, esters, acids and proteins) pentamoidae (hexenal, terpenes, benzyl alcohol) have been examined.

Publications

1. Aldrich, J. R., Blum, M. S., Hefetz, A., Fales, H. M., Lloyd, H. A., and Roller, P. Proteins in a nonvenomous defensive secretion: biosynthetic significance. Science 201: 452-454, 1978.

2. Aldrich, J. R., Blum, M. S., Lloyd, H. A., and Fales, H. M. Pentatomid natural products. Chemistry and morphology of the III-IV dorsal abdominal glands of adults. J. Chem. Ecol. 4: 161-172, 1978.

3. Lloyd, H. A., Evans, S. L., and Fales , H. M. Terpene alcohols of Helichrysum dendroideum II. Lloydia 21: 494-496, 1978.

4. Lloyd, H. A., Evans, S. W., Khan, A. H., Tschinkel, W. R. and Blum, M. S. 8-Hydroxyisocoumarin and 3,4-dihydro-8-hydroxyisocoumarin in the defensive secretion of the Tenebrionid beetle, Aspena pubescens. Insect Biochem. 8: 333-336, 1978.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01005-08 CH

PERIOD COVERED
October 1, 1978 - September 30, 1979

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:	R. Weber	Student	Univ. of Wisconsin
	T. Cantrell	Guest Worker	American Univ.

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemistry

SECTION
Chemical Structure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Crystallographic studies of compounds where full molecular dimensions are required to understand chemistry or physiological action. Development of techniques for atomic resolution studies of medium-size molecules

PHS-6040
(Rev. 10-76)

This report may be divided into four sections: studies of peptides and nucleotides, studies of morphinoid compounds, chemical problems, and technical developments.

1. Peptides and nucleotides

a) The synthetic cyclic polypeptide, (Prolyl-sarcosyl-(methyl)-alanyl-O-benzyl)-threanyl-valyl) crystallizes in $P2_1$ with two molecules in the asymmetric unit. It was of interest to establish the postulated all trans-conformation and also to correlate solid state with solution studies. The repeating unit has a weight of 1078 daltons and thus the problem is one of the larger direct methods determinations. The solution of the phase problem, while not routine, was carried out fairly rapidly and the structure has been refined to a R-factor of 3.7%, yielding band length with e.s.d.s of ca. 0.004Å. Comparison with solution studies is in progress with Dr. R. Highet (NHLBI) and Dr. A. Mauger (Washington Hospital Center). A paper on the project has been accepted for inclusion in the Sixth American Peptide Symposium (Washington, DC, June 1979).

b) Potassium and barium salts of guanosine meta phosphate. The structures of these compounds would be very useful for understanding the interaction of metal ions with the base. However, despite considerable experimental work, crystals have not as yet been grown which are large enough for Xray work. This work is being done in collaboration with Dr. T. Pinnavia (Michigan State Univ.).

c) Lupinosis factor. The compound responsible for Lupinosis, a serious disease in Australian sheep, has been isolated by Dr. Claude Culvenor (CSIRO). The constitution of the compound is presently unknown but it may contain peptide components and also chlorine. The molecular weight is large, ca. 1300 daltons, and the amount of the compound available very limited. The compound is crystalline but suitable crystals for X-ray work have not, as yet, been obtained.

2. Compounds related to morphinoid drugs

a) Colchiceine. Since colchiceine is only represented in the crystallographic literature by a very incomplete early study of a copper salt and also shows unusual solution spectrographic properties comparable with the morphine derivative reported by Iorio, Brossi and Silverton, it was of interest to carry out a full crystallographic study. The crystal structure was difficult to solve by standard techniques but yielded readily to techniques developed by the PI. The R-factor is 5.2% and the crystals prove to be an unusual double solvate of water and ethyl acetate. The water molecules control the packing and, since their presence was a surprise, re-evaluation of chemical results indicated that most preparations of colchiceine contain very firmly attached water molecules. The

ethyl acetate molecules are held mainly by packing forces and show some conformational or positional disorder, instanced by the R-factor which is somewhat high for this laboratory although regarded as respectable in many other places.

b) 7-keto dihydromorphinan. This compound was investigated with a view to helping in the elucidation of its solution chemical equilibria. Crystals are tetragonal, $I4_1$, and are unusual in that the space group is represented in the literature by only two solved structures (out of more than 13,000 reported structures). Structure solution was non-routine but the problem has now been refined to a R-factor of 3.1% despite complications caused by the fact that the repeating unit is two chemical molecules. The crystals are composed of molecules arranged in a very unusual hydrogen-bonded double helix. The product obtained in the solid state is apparently pure but the other solution compound would be unable to form the double-helical structure and thus probably crystallizes much less readily. The crystal structural results have prompted re-examination of the solution equilibria (by Dr. Kenner Rice, NIAMDD) and a consistent explanation is evolving.

3. Chemical Problems

a) 1-ethyl-3-carbomethoxy-1,3,3,4-tetra hydro-b-carboline (with Mr. R. Weber, University of Wisconsin). The chemical work on this compound produced conflicting assignments of stereochemistry. The problem was readily solved by crystallographic techniques.

b) Dibenzoylstilbene (with Dr. T. Cantrell, American U.). This structure was solved with a view to elucidating reaction stereochemical problems. The crystal structure readily explains the utilization of a chiral space group ($P2_1$) by a molecule possessing chemical symmetry.

c) Rohitukine from Amoora rohituka. The work, initiated last year, has been completed despite disorder problems and allows confident assignment of the structure of this new alkaloid.

4. Technical Developments

a) The X-ray diffractometer has been updated to the current state of the art allowing use of FORTRAN programming. Numerous technical difficulties arose in the update which occasioned considerable delays but problems now appear to be solved. The update should allow a much desired enhancement of the system to facilitate assignment of absolute configuration to molecules not possessing heavy atoms. Programming for this purpose has been initiated by the PI.

b) A recent paper (French & Wilson, Acta Cryst A34, 1218 (1978) cast doubt on the techniques used for treating "unobserved" reflections in X-ray data despite evidence from internal consistency in publications from this laboratory. Analysis of the theory allowed considerable simplification and made the techniques practical. However, comparison shows that present methods are entirely satisfactory although the new technique will be retained for compounds with problems of limited data.

c). The MULTAN-78 program has been implemented with modification by the PI to allow its use on larger problems than those for which it was originally designed. These modifications were necessary since many of our problems are considerably larger than average.

Publications:

1. Silverton, J. V. On the generation of 'magic integers'. Acta Cryst A34, 634 (1978).

2. Iorio, M. A., Brossi, A., Silverton, J. V. 7-oxo-desacetamido-colchiceine and 7-benzylimino-desacetamidecolchiceine: Two novel products from the base catalyzed reaction of (-)-N-benzylidene-desacetylcolchiceine. Helv. Chim. Acta 61, 1213 (1978.

3. Harmon, A. D., Weiss, U., and Silverton, J. V. The structure of Rohitukine, the main alkaloid of Amoora Rohituka (Syn. Aphanamixis Polystacha) Tetrahedron Lett. 1979, 721.

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5. Mauger, A. B., Stuart, O. A., Highet, R. J., and Silverton, J. V. Synthesis and Conformation of Cyclo(Thr-D-Val-Pro-Sar-MeALA). Sixth American Peptide Symposium, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01006-08 CH

PERIOD COVERED
October 1, 1978 - September 30, 1979

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 Chief, Structural NMR Section CH NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemistry

SECTION
Structural Nuclear Magnetic Resonance Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Dedrobatid Alkaloids: Six alkaloids of the Pumiliotoxin A series have been assigned structures based on nmr and mass spectral comparisons with the alkaloid 251D.

Steroids: The presence of an unusual intramolecular hydrogen bond has been demonstrated in seco-androstryn-3-ol-17-one. The structure of the microsomal metabolite of spironolactone has been confirmed by C-13 nmr.

Peptides: cyclo[Thr-D-Val-Pro-Sar-MeAla] has been examined by C-13 nmr to allow comparison of its solution confirmation with that in the crystal.

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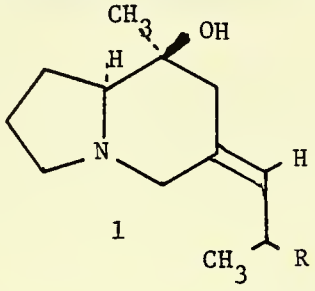
1. Dendrobatid Alkaloids

Studies with Dr. John W. Daly of NIAMDD and Dr. I. L. Karle of Naval Research Laboratories require the revision of previous structural conclusions, showing the dendrobatid alkaloid 251D to be 1A. Comparison of the proton and carbon-13 spectra of pumeliotoxins A and B to those of 251D show the



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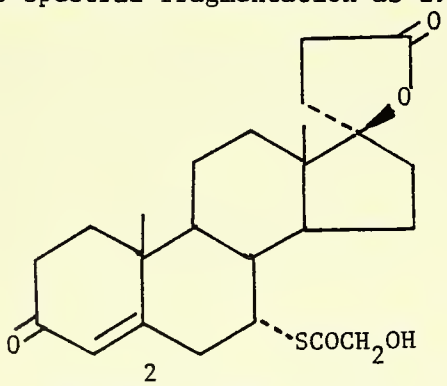
a. R is $CH_2CH_2CH_2CH_3$
b. R is $CH_2CH{=}C(CH_3)CHOHCH_2CH_3$
c. R is $CH_2CH{=}C(CH_3)CHOHCHOHCH_3$

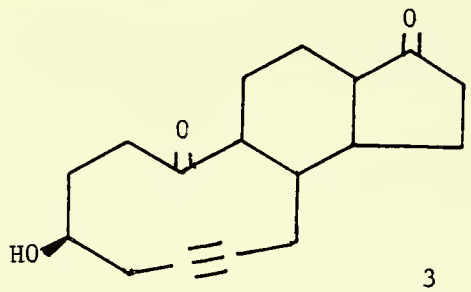
structure of these materials to be 1B and 1C, respectively. The unusual effects of this unique class of alkaloids upon the ion permeability of membranes assure that these structures will be the object of synthetic and drug development programs.

2. Spironolactone metabolites (with L. Pohl, LCP, NHLBI).

The proton and carbon-13 spectra of the metabolite of spironolactone produced by adrenal microsomes have been examined with the aid of the results of previous studies. They establish the structure inferred from mass spectral fragmentation as 2.



2



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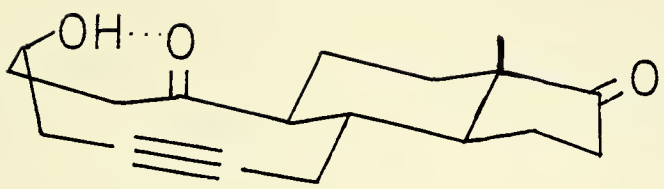
3. Secosteroid studies (with C. H. Robinson, Johns Hopkins University).

Earlier studies of secoaldosterones such as 3, which act as substrates or inhibitors of 3-keto-Δ-4 isomerase, revealed unusual dynamic behavior manifested as a broadening of the peak of C-1 in the carbon-13 spectrum. Studies of the effects of added polar solvents upon the C-13 nmr have now shown this behavior to depend upon an intramolecular hydrogen bond possible in the conformation 4. The interpretation is strikingly confirmed by the infrared spectra, which show the hydroxyl at frequencies characteristic of an intramolecular hydrogen bond, and the carbonyl involved as a doublet.



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4. (With A. B. Mauger of the Hospital Center and J. V. Silverton of this Laboratory). The C-13 and proton nmr spectra of the pentapeptide cyclo(Thr-D-Val-Pro-Sar-MeAla) have permitted a comparison of the conformation of the peptide in solution with that in the crystal. The most striking difference is the stronger hydrogen bond between proline and N-methylalanine in solution, which is very weak in the crystal.

5. (With H. Ziffer, NIAMDD). An investigation of the hydrolysis of 3,4-epoxycyclohexene by the copper-pyridine complex has shown the reaction to lack the stereospecificity shown in the hydrolysis of indene oxide.

Publications

1. Mauger, A. B., Stuart, O. A., Highet, R. J., and Silverton, J. V. Synthesis and conformation of cyclo(Thr-D-Val-Pro-Sar-MeAla), Sixth American Peptide Symposium, 1979.

2. Daly, J. W., Tokuyama, T., Highet, R. J., and Karle, I. L., A new class of indolizidine alkaloids from the poison frog, Dendrobates tricolor: X-ray analysis of 8-hydroxy-8-methyl-6-(2'-methylhexylidene) 1-azabicyclo [4,3,0]nonane. J. Amer. Chem. Soc., submitted.

3. Highet, R. J., Trager, W. F., Pohl, L. R., Menard, R. H., Taburet, A. M., and Gillette, J. R. Carbon-13 nuclear magnetic resonance studies of spironolactone and several related steroids. Steroids, submitted.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01008-08 CH

PERIOD COVERED

October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

The Use of Digital Computers in Problems in Biochemistry

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

PI:	G. W. A. Milne	Research Chemist	CH NHLBI
OTHER:	S. R. Heller	Computer Specialist	MIDSD EPA

COOPERATING UNITS (if any)

EPA, NBS, FDA, agencies in U.K., France, Switzerland, Holland, Germany, Hungary, Japan, Yugoslavia and the USSR

LAB/BRANCH

Laboratory of Chemistry

SECTION

Chemical Structure Section

INSTITUTE AND LOCATION

NHLBI-NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The NIH-EPA Chemical Information System, comprising 26 different components such as spectral data and bibliographic data bases is under systematic development in a project that involves the collaborative efforts of 51 different groups in the U.S., Europe, Japan and the U.S.S.R.

Components of this system include a mass spectral search system, a carbon-13 nmr search system, searchable data bases of x-ray diffraction data for organic and inorganic crystals and powders and a series of searchable files of toxicological data. A number of files concerning chemical pollution are in the system as well as searchable files containing chemical substances that are of concern to various agencies of the U.S. Government.

The entire system is being made available to the International Scientific Community via networked computers in the private sector. All who use the system do so on a fee-for-service basis.

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During this reporting period, operation of the NIH-EPA Chemical Information System, whose design and structure was described in the previous report, was continued. The CIS is now being used by some 220 subscribers, primarily in the U.S. and Europe.

Three main efforts have been undertaken during FY79. These are (1) addition of new components to the system, (2) improvement of the CIS user interface, and (3) design and implementation of logical links between components.

The components in the first release of the CIS (6/78) were the mass spectral search system, a mass spectrometric literature search system, the carbon-13 nmr search system, the substructure search system, the acute toxicity data search system, a search system for X-ray diffraction data for crystals and for powders, a statistical analysis package and a program for conformational analysis. Components that have subsequently been added to the CIS include a Technical Assistance Data System for Oil and Hazardous Materials (10/78) and an X-ray single crystal search system (6/79). In addition, the substructure search system has been modified to include a nomenclature search option. Components under development include an nmr literature search system, a search system based upon chemical citations appearing in the Federal Register and a thermodynamic properties search system.

Work has continued on the user interfaces and several new utilities have been incorporated into the public CIS. These include a program that reports to the user upon command the cost of the session, a CIS News system, a user status check program, and a series of programs that help users in the generation of off-line listings. In addition, a 24-hour, toll-free telephone has been established and handles about ten calls per day for either user assistance or general inquiries. An option within the CIS permits users to deposit complaints or other messages. Some eight messages are received by this route and responded to per week. About 10% of these relate to specific problems in the system, which is adjusted accordingly.

Many intercomponent links have now been installed in the CIS. As a result, it is now possible for a user to transfer upon command from most CIS components to most others. All search results are stored in files that can be accessed from any CIS component and thus it is feasible, for example, to search for a specific structure and then retrieve its mass spectrum, nmr spectrum and toxicity.

Publications:

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2. Milne, G. W. A., Zupan, J., Heller, S. R., and Miller, J. A.: A Substructure Oriented 13-C NMR Chemical Shift Retrieval System. Anal. Chim. Acta, 103, 141 (1978).

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11. Heller, S. R., and Milne, G. W. A.:The NIH-EPA Chemical Information System. Chapter 16 in ACS Symposium #94 (Ed. D. Schuetzle). American Chemical Society, Washington, D. C., (1979).

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13. Hartmann, K. N., Lias, S., Ausloos, P., Rosenstock, H. H., Schroyer, S. S., Schmidt, C., Martinsen, D. P., and Milne, G. W. A.: A Compendium of Gas Phase Basicities and Proton Affinities. NBS Interagency Report IR79-1777. NBS, Washington, D.C. (April 1979).

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01012-06 CH

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Amino Acid Sequence Determination of Polypeptides

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

PI:	J. J. Pisano	Head, Section on Physiological Chemistry	CH	NHLBI
Other:	M. Bledsoe	Research Chemist	CH	NHLBI
	T. Suzuki	Visiting Associate	CH	NHLBI

COOPERATING UNITS (if any)

None

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Laboratory of Chemistry

SECTION
Section on Physiological Chemistry

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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)

Using novel substrates synthesized in our laboratory, a new enzyme was discovered which splits the dipeptide prolylarginine from hippurylprolylarginine and prolylalanine from glycylprolylalanine. The enzyme has been tentatively named X-prolyldipeptidyl carboxypeptidase.

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PHS-6040
(Rev. 10-76)

Objectives: 1) Produce monospecific antibodies against the amino and carboxyl ends of bradykinin, angiotensin II, angiotensin III, and ranatensin. 2) Synthesize substrates to be used in the search for new dipeptidyl carboxypeptidases. 3) Discover new dipeptidyl carboxypeptidases to extend the usefulness of the GC-MS methods previously developed in our lab for polypeptide sequencing (Z01-HL-01012-05-CH).

Major Findings: 1) N-tert-butyloxycarbonyl-bradykinin was synthesized and coupled to chicken serum albumin by the active ester method using 1-hydroxybenzotriazole and 1-ethyl-3, 3-diaminoproplycarbodiimide. A poor yield was achieved at the coupling step. 2) The following peptides were synthesized by the active ester method using N-hydroxysuccinimide. Coupling was achieved with carbodiimide: p-Iodohippurylprolylarginine, p-iodohippurylprolylarginine methyl ester and p-iodohippurylarginine. The iodine was exchanged with [^{3}H] and the radioactive substrates provided a convenient and sensitive means to screen for enzyme activity. 3) An unstable enzyme which splits the glycyl-prolyl bond of hippurylprolylarginine was purified 600-fold from swine kidney in 0.63% yield. The enzyme is tentatively named X-prolyldipeptidyl carboxypeptidase.

Proposed Course: 1) To obtain a higher yield of coupling BOC-bradykinin to chicken serum albumin. When this is achieved, angiotensin II, angiotensin III, and ranatensin will be similarly coupled. Antibodies to the four immunogens will be raised in sheep. 2) To determine the substrate specificity and utility of X-prolyldipeptidyl carboxypeptidase in polypeptide sequence analyses.

Publications:

Krutzsch, H. and Pisano, J.J.: Preparation of Dipeptidyl Aminopeptidase IV for Polypeptide Sequencing. Biochemica et Biophysica Acta 576:280-289, 1979.

Nakamura, H., Zimmerman, C.L. and Pisano, J.J.: Analysis of Histidine-containing Dipeptides, Polyamines, and Related Amino Acids by High-Performance Liquid Chromatography: Application to Guinea Pig Brain. Analytical Biochemistry 93:423-429, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01016-09 CH

PERIOD COVERED
October 1, 1978 to September 30, 1979

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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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)

1. It appears that pepsin releases kinin only from kininogen molecules which contain the kinin moiety at the carboxyl end. 2. A radioimmunoassay for human urinary kallikrein is under development. Values obtained with this assay do not agree well with values for total kallikrein determined by the radiochemical esterase assay, yet the esterase can be removed from urine by immunoadsorption using an adsorbent containing monospecific antibodies to human urinary kallikrein. 3. A radioimmunoassay for urokininogen is under development. Values obtained with this assay do not agree well with values obtained for urokininogen based on bioassay of kinin released by trypsin or hog pancreatic kallikrein. 4. A radioimmunoassay for total plasma kininogen is under development. Values obtained with this assay do not agree well with values obtained by bioassay of kinin released with trypsin. High molecular weight kininogen in plasma can be accurately and reliably determined by radioimmunoassay (Z01 HL 01022-02 CH). High dietary salt appears to cause an elevation and administration of Fludrocortisone does not appear to change the plasma levels. One of four patients with hereditary angioedema had only half the normal level of plasma high molecular weight kininogen.

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Objectives: To improve procedures for the assay of components of the kallikrein-kinin-kininogen system and to use these procedures to establish the role of the system in health and disease.

Methods: Bioassay, radioimmunoassay, radiochemical assay, high performance liquid chromatography.

Major Findings:

Human Urinary Kallikrein: A radioimmunoassay (RIA) for human urinary kallikrein (HUK), capable of detecting 50 pg of antigen is under development. Human urine, human parotid saliva and purified human urinary prokallikrein (JVP 10-646-24) show parallel cross-reactivity to the standard. Immunoreactive antigen is present in human plasma and serum, but gives a logit-log plot which is not parallel to the standard. Human plasma kallikrein does not cross-react. Correlation of the RIA with the radiochemical esterase assay for free and total kallikrein is under investigation. In a study of 10 urines before and after gel filtration, there was good agreement between percent loss of kallikrein upon gel filtration (about 20%). However, the RIA values unexpectedly agree better with the free esterase than with total esterase. To demonstrate that esterase determined in the radiochemical assay is in fact HUK, urines were treated with antibody to HUK bound to Bio-Gel A-50m. Upon batch adsorption, 79-96% (mean 84 SEM 11) of the esterase was removed. The radiochemical assay of HUK and rat urinary kallikrein is very sensitive to salt concentration, especially in the range 0.01 to 0.1 M. The activity drops to about 30 percent the maximal activity when the salt concentration is 0.4 M KCl.

Urokininogen: Two methods for the assay of urokinogen are under investigation: 1) RIA of kinin released after incubation with hog pancreatic kallikrein or trypsin; 2) direct RIA of urokininogen. Interferences in the kinin assay caused by reagents, e.g., trypsin, Polybrene and Trasylol have been overcome by making the concentration of reagents identical in every RIA tube. Good agreement was obtained between the kinin values determined using trypsin and hog pancreatic kallikrein. Intra-assay reproducibility was also very good. However, the kininogen assay based on kinin release agreed with neither the direct RIA for total kininogen nor the RIA for high molecular weight kininogen, suggesting that released kinin arises from kininogen fragments with altered immunogenicity. The slope of the logit-log plot obtained in the RIA for total kininogen in urine is not parallel to the standard curve. Material in urine which cross-reacts in the HMW kininogen RIA was determined in seven normal volunteers on varying salt diets (samples provided by the Hypertension-Endocrine Branch). Preliminary results indicate that values are elevated in subjects on a high salt diet.

Action of Pepsin on Kininogen: Previous experiments showing that porcine pepsin releases methionyllysylbradykinin from human kininogen have been confirmed. The release is optimum at pH 1-2 but a second peak of activity occurs at pH 4-5.5 with about half the optimum. Trypsin releases kinin from all the kininogen types found in human plasma. Pepsin does not. Treatment of crude kininogen samples with carboxypeptidase B sometimes causes the complete destruction of the "pepsin substrate." Trypsin still releases kinin but not as much as before carboxypeptidase B treatment.

Human Renin and Plasma Prokallikrein: Human renin provided by Dr. Tadashi Inagami (Vanderbilt University) does not activate human plasma prokallikrein. However, renin activity could not be detected in the presence of prokallikrein. The prokallikrein sample either contains a renin inhibitor or an angiotensinase (which destroyed the renin product).

Plasma Kininogen: Preliminary estimates of total plasma kininogen, using a direct RIA under development, give about half the values determined by bioassay of releasable kinin. One reason for the lack of agreement is that the kininogen antibody does not quantitatively recognize the HMW kininogen-prokallikrein complex which exists in plasma. Attempts to dissociate this complex prior to RIA have, so far, been unsuccessful. RIA of HMW kininogen using a specific antibody gives values which agree with the bioassay for HMW kininogen. A prep B kininogen sample (10-205B-2), which was free of kinin-generating activity and from which free kinin had been removed by gel filtration, had a direct spasmogenic action on the rat uterus equivalent to 2-4% of the kinin content (1 µg kininogen ≈0.5ng bradykinin). Fludrocortisone administration to 7 normal volunteers did not change their levels of HMW kininogen as measured by RIA. One patient with hereditary angioedema had half the normal level of antigen, the lowest value ever seen apart from congenitally deficient subjects. Two patients with Bartter's Syndrome had normal levels.

Proposed Course: An explanation will be sought for the lack of a good correlation between total kallikrein determined by the radiochemical esterase assay and the RIA. The effect of salt concentration on the activity of kallikrein will be determined using the esterase assay and the bioassay for kinin release from kininogen.

Urokininogen: Material in urine which cross-reacts with antibodies raised to low molecular weight and high molecular weight kininogens will be characterized. Stability, cross-reactivity (parallelism) and physiochemical properties will be studied. Explanations for the lack of agreement between urokininogen determined by assay of kinin release with that determined by direct RIA will be sought.

Plasma kininogen: Development of an RIA for total kininogen will be continued and an explanation sought for the lack of agreement between RIA and bioassay.

Action of Pepsin on Kininogen: Better documentation is needed to prove that pepsin acts only on kininogen molecules which contain the kinin moiety at the carboxy terminus. Kininogen species which are substrates for pepsin will be identified.

Publications:

Pisano, J.J., Corthorn, J., Yates, K., and Pierce, J.V.: The Kallikrein-Kinin System in the Kidney. Contributions to Nephrology 12:116-125, 1978.

Vinci, J.J., Zusman, R.M., Izzo, J.L.Jr., Bowden, R.E., Horwitz, D., Pisano, J.J., and Keiser, H.R.: Human Urinary and Plasma Kinins: Relationship to Sodium-Retaining Steroids and Plasma Renin Activity. Circulation Research 44:228-237, 1979.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01018-22 CH

PERIOD COVERED
October 1, 1978 to September 30, 1979

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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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

K_m and k_{cat} values were determined for human Hageman factor (factor XII) fragment(s) (HF_f) with plasma prokallikrein and six synthetic substrates. A monospecific antiserum to HF_f was obtained in sheep. New plant inhibitors of HF_f, plasma and urinary kallikreins, factor X, and thrombin were detected.

SDS-polyacrylamide gel electrophoresis of the four main electrofocusing forms of human plasma prokallikrein gave a band at M_r=84,000 for each. The pI 9.3 form also had an equally strong band at M_r=80,000, while the pI 9.1 and 8.8 forms had progressively weaker bands at this M_r. HF_f treatment gave the same pattern. Sheep antiserums to the pI 8.8 prokallikrein and to the kallikrein derived from it were monospecific except for some anti-IgG in the antiserum to prokallikrein.

Human plasma kininogens, prepared by immunoaffinity chromatography in the presence of Polybrene and several serine protease inhibitors, gave a DEAE-cellulose chromatographic profile very similar to that found previously for kininogens purified in the presence of Polybrene only.

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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 proteases (kallikrein, Hageman factor, plasmin, 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 proteins, affinity adsorbents, and assay methods to studies of normal and pathological states in man and other primates.

Major Findings:

1. Human Plasma Kallikrein and Prokallikrein. The four major forms (pI 8.5, 8.8, 9.1, and 9.3) of human plasma prokallikrein described previously (Z01 HL 01018-20, 21 LC) were subjected to SDS-PAGE (polyacrylamide gel electrophoresis) in the absence of reductant. A strong band at M_r=84,000 characterized all four forms, but the pI 9.3 form also had an equally strong band at M_r=80,000, while the pI 9.1 and 8.8 forms showed progressively weaker bands at this M_r, and the pI 8.5 form showed no discernible trace of this band. The same pattern was observed after activation of the four forms by HF_f. Mandle and Kaplan (J. Biol. Chem. 252, 6097-6104, 1977) obtained a purified plasma prokallikrein fraction which gave two bands on SDS-PAGE gels corresponding to M_r's of 88,000 and 85,000. However, they did not separate these forms nor did they observe the multiple forms seen on electrofocusing by us and by Laake and Venneröd (Thromb. Res. 4, 285-302, 1974).

Sheep antiserums were prepared with good titers against the pI 8.8 form of plasma prokallikrein and the active kallikrein derived from it by treatment with HF_f. The antiserum to plasma kallikrein is monospecific, while that to the proenzyme contains some antibody to IgG, when tested against normal and kallikrein-deificient plasmas by the Ouchterlony method.

2. Hageman Factor Fragment(s) (HF_f).

(a) Kinetics. Kinetic properties of HF_f were determined with its natural substrate, human plasma prokallikrein, and with several synthetic substrates, as summarized in the following table.

kaolin-activated HF from acting on factor XI. The K_i of CHFI (pI 6.3 form) with HF_f was determined to be 2.4×10^{-8} M, using the chromogenic substrate D-Pro-Phe-Arg-pNA (S-2302).

3. Plant Inhibitors of HF_f, Plasma Kallikrein, Plasmin, Thrombin, Factor Xa, Human Urinary Kallikrein, and Trypsin. A wide variety of plant materials--beans, seeds, flower bulbs, etc.--were screened for inhibitors of HF_f, plasma kallikrein, and bovine trypsin. The most promising extracts were chromatographed on DEAE-cellulose and sometimes further purified by gel filtration or electrofocusing. Some of these chromatographic fractions were further screened for inhibitory activity toward α-thrombin, plasmin, factor Xa, human urinary kallikrein, hog pancreatic kallikrein, and bovine α-chymotrypsin. Iris bulbs and the seeds from certain members of Cucurbitaceae contained inhibitors of HF_f and trypsin but not of plasma kallikrein or the other serine proteinases mentioned above. The M_r's of the pumpkin seed HF_f inhibitor and iris bulb HF_f inhibitor (heterogeneous by DEAE-cellulose chromatography) were estimated to be 2,000-3,000 and 10,000, respectively. Other sources of HF_f inhibitors were peanuts and tulip, lily, hyacinth and grape hyacinth bulbs, although these inhibitors also inhibited plasma kallikrein. Their M_r's were estimated to be 7,000, 25,000, 25,000, 7,000, and 25,000, respectively, although multiple forms were found by DEAE-cellulose chromatography. Lily bulbs contain an inhibitor (M_r=25,000) for thrombin which does not inhibit Xa, while hyacinth and grape hyacinth bulbs contain inhibitors for factor Xa which do not inhibit thrombin. Inhibitors of human urinary kallikrein were found in apple seeds, lily bulbs, avocado pits, and grape seeds. The apple seed inhibitor does not inhibit trypsin.

4. Human Plasma Kininogens. A new procedure for the purification of kininogens was devised (old procedure: NHLI-284, 1974). Platelet-free CPD plasma, made to 0.1% NaN_3, 1mM DFP, 0.005% STI and LBI, 5 mM benzamidine-HCl, 0.5 mM N-ethylmaleimide (NEM), 0.01% Polybrene at 25°, was cooled to 6° and passed over a column of anti-kininogen-agarose. The column was washed extensively with buffers of different ionic strength and pH, all containing 0.25 mM DFP and 5 mM benzamidine-HCl. The adsorbed kininogens were eluted with 8.0 M guanidine-HCl/2mM NaP_i/ 1mM EDTA/ 0.5 mM DFP/ 0.1 mM NEM, pH 4.5, followed by 0.25 mM DFP/ 2 mM benzamidine-HCl/ 1 mM EDTA/ 0.01% NaN_3, pH 6.7, to wash off the guanidine. The eluate was made to 4.0 M with solid guanidine-HCl and dialyzed twice against 0.1 mM DFP/ 2.5 mM benzamidine-HCl while stirring at 6°. The dialyzed eluate was made to 0.025 M NaP_i/ 1 mM DFP/ 2.5 mM benzamine-HCl, pH 6.1, and adsorbed to a column of high-capacity DEAE-cellulose, then eluted with a linear gradient of 0.05 to 0.30 M NaP_i, pH 6.0, in the presence of 2 mM DFP and 0.25 mM benzamidine-HCl. The elution profile (A_{280} and kininogen activity) was quite similar to that previously obtained, except that the B2 and B3 peaks were not so well resolved as before. However, the present B4 peak (HMW kininogen) had 1.5-2.0 times the specific activity found for the previous B4 fraction.

Substrate	Optimum pH	Spec. Activity[1]	K_m, mM	k_{cat}, sec^{-1}	k_{cat}/K_m, $M^{-1}sec^{-1}$ x 10^{-5}
Plasma prokallikrein		4.5	0.00086	2.6	30
Tos-Arg-OMe	8.0-8.5	5.1	5.1	12	0.024
Bz-Arg-OEt		10.7	0.37	9.4	0.25
Ac-Phe-Arg-OEt		77	0.11	46	4.2
D-Pro-Phe-Arg-pNA (S-2302)	7.5	26	0.013	14	10.8
Bz-Ile-Glu(γ-OR)-Gly-Arg-pNA (S-2222)	8.0	33	0.18	23	1.28
D-Val-Leu-Arg-pNA (S-2266)		16			
Tos-Gly-Pro-Arg-pNA		22			
Z-Gly-Pro-Arg-pNA		4.2			
D-Phe-Pip-Arg-pNA (S-2238)		11			
<Glu-Gly-Arg-pNA (S-2444)		10			
D-Val-Leu-Lys-pNA (S-2251)		<0.1			
Bz-Phe-Val-Arg-pNA (S-2160)		<0.1			
Bz-Pro-Phe-Arg-pNA		<0.1			
Bz-Arg-MCA[2]		<0.1			
Z-Phe-Arg-MCA		<0.1			
Pro-Phe-Arg-MCA		3.3	0.054	1.9	0.34
Boc-Val-Pro-Arg-MCA		0.9			
Boc-Ile-Glu-Gly-Arg-MCA		1.1			
Z-Lys-SBzl		8.0	1.7	17.9	0.11

[1] μmol min^{-1} mg^{-1} in 0.05 M Tris-HCl, pH 8.0, at 25°. Substrate concentrations: 0.012 mM prokallikrein; 0.5 mM synthetic substrate.

[2] 4-methyl coumaryl-7-amide

Substrate inhibition was observed with Bz-Ile-Glu(γ-OR)-Gly-Arg-pNA (S-2222), while Bz-Phe-Val-Arg-pNA (S-2160) and Bz-Pro-Phe-Arg-pNA were inhibitory when using D-Pro-Phe-Arg-pNA (S-2302) to measure remaining HF_f activity.

(b) Immunology. After several boosts, the sheep previously immunized (see Z01 HL 01018-21 LC) with excised PAGE disc gel bands of HF_f produced an antiserum giving a single, strong precipitin line with the immunogen (at 0.10 A_{280}) as well as with 5-fold concentrated human plasma and the other DEAE-cellulose peak of HF activity (see Z01 HL 01018-21 LC). Since no precipitin line was seen with 3.5-fold concentrated Hageman plasma applied twice to the Ouchterlony well, the antiserum is presumably monospecific.

(c) Corn HF_f Inhibitor (CHFI). The HF_f inhibitor from corn, CHFI (see Z01 HL 01018-21 LC) markedly retarded the partial thromboplastin time (PTT) of normal plasma. Also, correction of the delayed PTT of Hageman factor-deficient plasma by added HF_f was abolished by CHFI, suggesting that CHFI inhibits

Significance to Biomedical Research and the Program of the Institute: See Z01 HL 01018-20 LC

Publications:

Yamada, T., Wing, D.A., Pierce, J.V., and Pettit, G.W.: Turnover of human and monkey plasma kininogens in rhesus monkeys. J. Clin. Invest. 63:45-52, 1979.

Pisano, J.J., Corthorn, J., Yates, K., and Pierce, J.V.: The Kallikrein-Kinin System in the Kidney. Contributions to Nephrology 12:116-125, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01022-02 CH

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Physiological Significance of Human High Molecular Weight Kininogen

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

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None

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.9	.9	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A radioimmunoassay (RIA) for human high molecular weight kininogen (HMWK) has been improved and more firmly established. Extensive cross-reactivity studies have been performed. The assay has been applied to 17 normal plasmas and showed excellent correlation with bioassay. Gel filtration of normal plasma showed no antigenic fragments. Seven plasmas reportedly deficient in HMWK also have been studied using the RIA. Antigen has been detected in urine.

PHS-6040
(Rev. 10-76)

Objectives: To determine the physiological significance of high molecular weight kininogen (HMWK). To more firmly establish a sensitive, selective, direct radioimmunoassay (RIA) for human HMWK.

Major Findings: Two modifications of the RIA (Z01 HL 01022-01 CH) have improved its sensitivity and reliability. After gel filtration of iodinated HMWK, the peak fraction is now re-chromatographed and this peak fraction used in the assay. The maximum binding of the label has been raised from ∿65% to 85-90%. The double antibody precipitation step has been improved by inclusion of sheep IgG as a carrier. With these two modifications it is possible to use the antibody to HMWK at a final dilution of $^{1}/_{400,000}$ and to detect as little as 150 pg of antigen. The intra- and inter-assay coefficients of variation are now 2% and 1.5%, respectively. Recovery of HMWK added to 6 plasmas was 97.7 ± 1.8%.

In light of the increased sensitivity and reliability of the assay, cross-reactivity studies were repeated and extended. Bradykinin and human plasma kallikrein showed no cross-reaction while monkey HMWK showed identity to the human antigen. Of a range of human low molecular weight kininogens tested (Project No. Z01 HL 01016-06 LC) only B3.2 showed any significant cross-reaction (2%). This antigen gave a logit-log plot which was not parallel to the standard, indicating that B3.2 did not share all the antigenic determinants possessed by HMWK.

When plasma was treated with purified human plasma kallikrein, no change in antigenic content was seen, indicating that kinin-free kininogen and/or breakdown fragments could be expected to cross-react. Hence, RIA measurements in the presence of such fragments may lead to spuriously high values. However, assay of 17 normal plasmas gave a level of 90.8 ± 2.5 µg/ml ($\bar{x}$± SEM) while a bioassay of the same samples, based on specific release of kinin from HMWK by purified plasma kallikrein, yielded a level of 90.2 ± 2.8 µg HMWK/ml ($r = 0.83$, $p<0.001$). In neither assay was any significant sex difference observed. The excellent correlation between RIA and bioassay suggests that no significant non-functional kininogen exists in normal plasma, presumably indicating a low turnover of HMWK in normal individuals and/or a high catabolic rate for any breakdown fragments. Gel filtration of normal plasma also showed no evidence of any free antigenic fragments.

RIA measurements were also performed on 7 plasmas reportedly deficient in HMWK. Williams, Dayton, San Francisco and Flaujeac plasmas all showed no significant cross-reaction while Fitzgerald, Reid and Detroit plasmas showed 1.0, 2.5 and 3.5% of normal antigenic levels, respectively.

Further studies on the antigen detected in urine revealed that using freshly voided urine, a line parallel to the standard was obtained while with overnight or 24-hour urines parallelism was lost. This suggests that intact light chain (the unique moiety of HMWK) may initially be cleaved but may then be susceptible to hydrolysis by urinary proteases.

Significance to Biomedical Research: The RIA for HMWK should allow for the first time an assessment of the involvement of kininogen and plasma kallikrein in several normal and pathological states in man.

Proposed Course: To apply the assay to various clinical conditions in which the plasma kallikrein-kinin system is selectively activated in order to gain insight into the physiological role of the system. Continued bioassay correlations will be performed to verify that the RIA is measuring functional HMWK and, in any cases where the assays no longer correlate, the RIA may be used to monitor the fate of non-functional kininogen. Characterization and development of an assay for the cross-reacting protein in urine will be undertaken.

Publications:

Proud, D., Pierce, J. V., Peyton, M. P. and Pisano, J. J.: A radioimmunoassay for human high molecular weight kininogen. Fed. Proc. 38:686, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01023-01 CH

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Biochemical Events in the Expression of Hormonal Activity: Role of Proteases

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)

Arginine vasopressin (AVP) added to the serosal bath of an isolated toad urinary bladder preparation in addition to increasing water flow caused a dose-dependent release of proteinase(s) into the mucosal but not the serosal fluid. Low molecular weight serine proteinase inhibitors added to either the serosal or mucosal fluid significantly inhibited the AVP-induced water flow and the elaboration of the proteinase(s). Trypsin added to the serosal (but not the mucosal) surface induced an AVP-like water flow without increasing the proteinase activity in the mucosal fluid. cAMP also induced water flow without a detectable increase in proteinase activity in the mucosal fluid. One inhibitor tested so far, which effectively inhibited AVP and trypsin, did not alter the cAMP-induced water flow.

PHS-6040
(Rev. 10-76)

Major Findings: Water flow and proteinase activity have been measured in the isolated urinary bladder of the toad. The proteinase was determined by a radiochemical esterase method employing (^{3}H) -tosylarginine methylester (TAME). Arginine vasopressin(AVP) added to the serosal bath in addition to increasing water flow caused a dose-dependent release of a TAME esterase into the mucosal but not the serosal fluid. The serine proteinase inhibitors, trasylol and leupeptin, added to the serosal fluid significantly inhibited the AVP-induced water flow and esterase activity. Added to the mucosal side, leupeptin but not trasylol also was inhibitory. Several experimental peptide blockers (blockers of bradykinin and AVP in the isolated rat uterus and guinea pig ileum and inhibitors of some proteinases) provided by KABI are equally effective against both activities. These include S2440, S2441, S2457, and, to a lesser extent, S2302. These agents were active on the mucosal and the serosal side. Also effective at 10^{-5}M on both sides was the chymotrypsin inhibitor TPCK. The trypsin inhibitor TLCK was inactive. However, trypsin (but no DFP-treated trypsin nor chymotrypsin) added to the serosal bath stimulated water flow in a dose-dependent manner. Trypsin was inactive on the mucosal side (chymotrypsin has not been tested). Addition of S2440 to the mucosal but not the serosal side significantly inhibited the trypsin-induced water flow. cAMP induces water flow without a detectable increase in esterase activity in the mucosal fluid. S2440 added to either the mucosal or serosal fluid did not alter the cAMP-induced water flow.

Significance to Biomedical Research and the Program of the Institute: Knowledge of the chemical events which follow the attachment of a hormone to its receptor in a target organ will provide a better understanding of how hormones act and how their hormonal activity and actions may be controlled. This knowledge would be of interest in situations where there is a hormonal deficiency or undesired activity.

ANNUAL REPORT OF THE
LABORATORY OF EXPERIMENTAL ATHEROSCLEROSIS
NATIONAL HEART, LUNG AND BLOOD INSTITUTE
October 1, 1978 through September 30, 1979

The lesions of atherosclerosis are discrete and appear to have a characteristic topographic distribution in a given animal species, including man. A typical lesion consists of a localized region of intimal thickening characterized histologically by smooth muscle cell hyperplasia, connective proliferation, and increased lipid deposition, including cholesterol and cholesteryl ester. Although the role of smooth muscle cell proliferation in atherogenesis has received considerable attention recently, there is no evidence that proliferative changes in the absence of lipid deposition contribute to the morbidity of this disease. In fact, under experimentally induced conditions, intimal smooth muscle hyperplasia is a reversible process. Accordingly, our efforts have focused on the events leading to intimal lipid deposition since this occurrence appears to be most closely correlated with the lethal consequences of the disease. Our major activities have been directed toward development of 1) topographic analytic techniques for study of the spatial patterns of the disease, 2) methodology to study the transport processes related to intimal lipid deposition, 3) definition of the nature and location of the barriers to transarterial lipid transport, and 4) detailed study of the altered lipoprotein metabolism associated with atherogenesis.

Topography. The topographic distribution of atherosclerosis is studied from opened arterial specimens that have been stained with Sudan IV. Regions of increased intimal lipid deposition appear as sudanophilic patches on the opened arterial tree. The sudanophilic pattern that appears early in the disease is characterized as simple fatty streaking; however, in a matter of months certain areas in this pattern evolve into typical atheromatous plaques. This is particularly true of the coronary arteries, lower abdominal aorta, and larger peripheral arteries. The predictability of these patterns at given locations suggests that appropriate statistical approaches using modern image processing techniques will permit study of the sequence of events leading from the "earliest" lesion to the complicated atheromatous plaque. Preliminary results have allowed certain crude correlations: The locations of altered endothelial structure and physiology (perhaps secondary to local hemodynamic events) appear to correlate with regions of increased lipid deposition as detailed in previous reports.

Therefore we have pursued development of automated image processing techniques to place these types of correlations on a firmer scientific footing. This approach is made possible by standardized necropsy and formating procedures that we have developed. Vessels are opened and pinned flat in a standard format on which it is possible to identify from 10-50 invariant anatomical landmarks to be used as fiducial points. The x-y coordinates of each are measured and taken in sets of three to calculate the six coefficients necessary for a linear transformation of the data contained within the triangle bounded by the three fiducial points onto the corresponding triangle of a standardized coordinate system. The necessary scanning procedures

(using microdensitometry from photographic transparencies or using television from the opened specimens at necropsy) have been worked out with collaborators from the Division of Computer Research and Technology and the Biomedical and Instrumentation Branch. The necessary image processing software and statistical analytic algorithms have been developed and checked with pilot studies. This interdisciplinary effort should soon be operational and will represent a major advance in our ability to carry out the indicated topographic correlations.

Transport. As described in previous annual reports, we have demonstrated that the principal barrier to the flux of plasma substances into the arterial wall is the endothelial surface. We have previously demonstrated that the permeability and structural characteristics of the endothelial surface are exquisitely sensitive to the intensity of shear stress created by the adjacent blood flow and, of even greater interest, that the endothelial surface appears to compensate both structurally and functionally for altered stress patterns with time, provided the stress is unidirectional. Study of these mechanisms by which this surface responds to changing directions of stress, however, has been experimentally intractable to date. Accordingly, this year we have put considerable effort into developing an in vitro system in which it is possible to study acute changes of endothelial permeability, not only in response to changes in the magnitude but also to changes in the direction of the imposed shear stress. These studies have been carried out using the canine descending thoracic aorta. Although preliminary results indicate that the permeability of the endothelial surface does appear to increase with a change in the direction of shear stress, as well as magnitude, these experiments have been technically disappointing because of the enormous variability from animal to animal in the control level of endothelial permeability. This unanticipated problem is under separate study to seek ancillary correlations with the genetic background, dietary background, etc., of these animals.

We have continued to study the albumin-Evans blue dye (EBD) ligand system as a prototype for macromolecular transport across arterial tissue. Background studies have been described in previous annual reports. This year we have made important advances in our understanding of detailed transvascular transport in arterial tissue, as well as in the development of new methodology, for the discrete measurement of concentration distributions across the arterial wall. The transport of albumin across the arterial wall is governed by two processes, diffusive and convective. In the absence of a pressure across the wall and in the absence of an endothelial surface, the uptake of radiolabeled albumin was shown to follow (to a first approximation) a "square root of time law," indicating that the wall was behaving similar to a "homogeneous slab" model for diffusion. In the presence of a normal endothelial surface, the uptake was found to be linear with time. Moreover, in a period of one hour, about five times less albumin enters the normal wall than enters the deendothelialized wall. These data were consistent with those mentioned earlier, indicating that the endothelial surface acts as a discrete but very large barrier to the transport of protein from the plasma phase to the arterial phase.

In vitro studies by us to examine the role of increased wall stretch on the transport processes have shown that the permeability of the endothelial surface is increased by increased stretch in the absence of an increased pressure. In the case of the deendothelialized arterial wall, studies this year have shown that the transport processes of the intimal-medial portion of the system are virtually uninfluenced by the degree of wall stretch. In view of the anisotropic and microscopically heterogeneous composition of the intimal-medial tissue, these results are surprising and are being repeated.

Other studies this year, designed to study the role of increased transmural pressure on transport processes, showed that pressure itself can significantly increase the transport of labeled proteins across the deendothelialized intimal-medial preparation. A transmural pressure of ~100 mm Hg that is imposed across the wall appears to add a significant convective force to transport over and above the diffusive forces, thereby increasing the uptake of labeled albumin. A special technique was devised whereby the influence of the separate driving forces could be separated and measured. It was found that in the deendothelialized wall, convective transport was of the same order of magnitude as the steady-state diffusive transport; thus, for the first time, it was shown that an increased arterial pressure increases the influx of plasma substances into the arterial wall (as has been assumed from clinical observations but never measured before). Similar studies are currently being done for the intact endothelialized artery in conjunction with those mentioned above (to study the role of stretch). The significance of these studies is that they allow quantitation of the role of both altered pressure states as well as altered diffusive states so that we now have another tool for gaining deeper insight into the mechanisms of atherogenesis and its relationship to hypertension.

Considerably more information regarding transport processes through arterial tissue can be gained by studying the discrete concentration of a particular radiolabeled protein across the arterial tissue under experimentally imposed constraints of time, concentration, and manipulated physiologic and pharmacologic states. Until now, technology for this has been possible only under very special experimental circumstances. We have developed methodology so that these important measurements can be done now in a variety of in vitro and in vivo experimental configurations and with a high degree of spatial resolution. To do this, quantitative autoradiographic techniques were developed in which the local concentration of silver in the developed autoradiograph is measured by scanning across the micrograph with an electron microprobe. These experiments demonstrated that the relationship between the concentration of radiolabeled protein at any point in the wall is directly proportional to the signal from the electron microprobe. The initial application of this new methodology has been to determine the distribution of radiolabeled albumin across deendothelialized aortic and iliac arterial walls in the quasi steady state and in the absence of a transmural pressure. The results of these studies have shown that the partition coefficient for radiolabeled albumin is nominally constant across the arterial wall, that the principle of superposition applies to diffusive transport of albumin through aortic tissue, and that the coefficient of diffusion for albumin in arterial tissue varies at certain sites across the wall in relation to various histologic structures. The significance of these studies, apart from the

development of an experimental tool that should be applicable to other tissue systems for measurement of radiolabeled protein distributions, is that we can begin to study the parameters of macromolecular transport across the arterial wall in sufficient detail that, for the first time, it should become possible to model these processes by physical and mathematical laws. This should permit inferences regarding detailed distributions of various macromolecules, such as atherogenic precursors, across the arterial wall from relatively simple measurements of uptake. Secondly, since this methodology provides access to detailed events within the wall, it opens the way to study of the equally important transport processes of cholesterol-rich metabolites out of the wall. Thus we have made significant progress toward developing the experimental and conceptual tools necessary for further definition of the nature of the unbalanced set of transport and metabolic processes associated with the intimal lipid deposition of atherosclerosis.

Lipoprotein and arterial metabolism. Studies have continued on dietary-induced atherosclerosis in dogs, miniature swine, and Patas monkeys. These studies have allowed us to define the metabolic parameters necessary to produce arterial disease with characteristics resembling human atherosclerosis and have also provided new insights into some of the processes involved in the genesis of this important disease. The source of the dietary fat, fed in association with cholesterol, has been linked to the type, distribution, severity, and complications of the atherosclerosis. Cholesterol-rich diets which contain saturated fats, such as beef tallow, cause severe atherosclerosis that is frequently associated with thrombosis, particularly in dogs. Diets which contain unsaturated fats, e.g., cottonseed oil, result in a much less fulminant disease and no thrombosis. Our studies provide for the first time a reproducible model in which to study the role of the hemostatic processes, including platelet physiology, in the initiation and/or progression of the disease process. Changes in platelet membrane fluidity resulting from altered fatty acid composition and cholesterol content is thought to be responsible for sensitization of platelets to aggregation and thrombus formation. Studies are in progress to define the lipid composition of the membranes of platelets from animals on the various dietary protocols and to attempt to correlate these findings with changes in platelet function. Preliminary studies suggest that platelets from dogs on a saturated fat-cholesterol diet are promptly "sensitized" to aggregation. Platelets from dogs on an unsaturated fat-cholesterol diet react more normally.

Studies designed to characterize the plasma lipoproteins associated with cholesterol feeding in dogs, miniature swine, rats, rabbits, and Patas monkeys have led to the identification of certain consistent features of the atherogenic hyperlipoproteinemia. These include: 1) the production of a lipoprotein similar to the B-VLDL which occurs in human Type III hyperlipoproteinemia; 2) an increase in the low density lipoproteins and the intermediate lipoproteins which may arise from VLDL catabolism; and 3) the identification of a previously undescribed lipoprotein referred to by us as HDL_C. Associated with all of these lipoproteins is an increased prominence of a specific apoprotein protein, the "arginine-rich" apoprotein. Characterization of these lipoproteins and their apoproteins in the various species mentioned above is now completed. Studies are in progress to determine the

origin and metabolic fate of these lipoproteins. Particular emphasis is being placed on the role of the "arginine-rich" apoprotein and the HDL_C in the regulation of cholesterol metabolism.

One of the important characteristics of the HDL_C, that is also shared with LDL, is the ability to be bound and degraded by skin fibroblasts and arterial smooth muscle cells in culture. It has now been established that LDL and HDL_C are specifically bound to the same high-affinity cell surface receptor site whereas the typical HDL are not. Considerable progress has been made in defining the chemical nature of the recognition site on the lipoproteins responsible for binding to the receptor. The following conclusions, as reviewed in the progress reports, are now possible: 1) the HDL_C and LDL bind to the same receptor site, 2) the protein moieties of the lipoproteins determine the specificity for binding, 3) both the B apoprotein of LDL and the arginine-rich apoprotein of HDL_C are capable of binding to the receptor, 4) a similar positively charged region or structural sequence of the protein may be shared in common between the B and arginine-rich apoproteins, and 5) a limited number of arginyl and lysyl residues are functionally significant residues in the lipoprotein recognition site. The precise nature of the chemical reaction of specific arginines and lysines in the lipoprotein recognition site with the cell surface receptors is being actively investigated. Selective chemical modification of a limited number of lysyl and arginyl residues has proved to be a highly useful procedure with which to probe the metabolic role of certain plasma lipoproteins. We have shown that modifications of LDL which alter the charge on the lysines of apo-B result in a rapid, acute clearance of these lipoproteins from the plasma by Kupffer cells of the liver. Furthermore, these modified lipoproteins are internalized and degraded by macrophages in culture. It is reasonable to speculate that such modification may occur in nature and be responsible for clearance of certain lipoproteins. Studies along these lines will be continued. Elucidation of these mechanisms may ultimately provide clues as to how to prevent cellular cholesterol deposition.

Our detailed understanding of the cholesterol-induced lipoproteins of the various animal species has provided a strong basis for the investigation of the effects of cholesterol feeding in man. Studies are in progress to supplement the diet of men and women (20-45 years of age) with 4 to 6 eggs per day for 3 to 4 weeks and to characterize in detail the changes in the plasma lipoproteins. Results obtained in eleven subjects studied to date indicate that the equivalent of the HDL_C occurs in people after cholesterol (egg) feeding in a subfraction of the d=1.063-1.125 ultracentrifugal fraction. Furthermore, this lipoprotein appears following cholesterol feeding with or without an elevation of the plasma cholesterol level. This subfraction has characteristics similar to those described for the HDL_C of the lower species and binds to the cell surface receptors. It has now been possible to isolate and quantitate the HDL_C induced by cholesterol feeding in man using heparin affinity column chromatography.

CONCLUDING COMMENT

We have made important advances in our understanding of lipoproteins and their role in atherogenesis in the experimental animal and have discovered

important similarities between these processes and those occurring in man. We have developed new insights into the macromolecular processes associated with transport in the arterial wall. We have developed new technology bringing us closer to our goal of studying the physical and biochemical processes in situ associated with atherogenesis in the living tissue system. This will allow us to interpret better the significance of the histological changes that evolve in man and animal during atherogenesis and the relevance of observations on cellular metabolism studied in artificial systems such as tissue culture. Finally, we have made a major advance toward our goal of being able to interpret the messages hidden in the topography of this important disease process.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER: Z01 HL 02803 06 EA

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
Study of arterial transport processes in an in vitro support system

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

PI:	D.L. Fry	Chief, Laboratory of Experimental Atherosclerosis	EA NHLBI
Other:	R.W. Mahley	Head, Comparative Atherosclerosis and Arterial Metabolism Section	EA NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION
Vascular Physiology Section

INSTITUTE AND LOCATION
NIH/NHLBI-DIR, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	.2	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The purpose of this study was to measure the uptake and the concentration distribution of radiolabeled albumin across the deendothelialized aortic wall at various selected times after experimentally imposed states of static stretch.

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PHS-6040
(Rev. 10-76)

PROJECT DESCRIPTION

Objective: To examine the change in the arterial uptake of radiolabeled albumin under controlled conditions of stretch and time.

Methods: Aortas from dogs were quickly removed under special conditions and placed in a specially designed device in which varying degrees of circumferential and longitudinal stretch could be imposed on the arterial tissue. Another special device containing matching chambers was applied to the arterial surface so that the chemical milieu of the intimal and adventitial surfaces could be manipulated in accordance with experimental protocol. Radiolabeled albumin solutions ionically and oncotically matched to plasma were placed in the intimal chambers for exposure durations of 32 minutes at various prescribed times following imposition of different states of stretch.

Major Findings: It was found that the 32-minute uptake of radiolabeled albumin by the intimal-medial preparation varied slightly both with stretch as well as with time. In the period immediately following altered vascular stretch, it was found that increased stretch was associated with a decreased uptake whereas decreased stretch was associated with an increased uptake. Over the ensuing 2-hour period, however, when another 32-minute exposure period was imposed, an inverse pattern was observed. The changes in uptake were surprisingly small. In no case did the change in uptake exceed 5% even though the range of vascular stretch was ±30%, values designed to span those encountered in shock and hypertension (0 and 200 mm Hg).

Significance: Convincing epidemiologic evidence suggests that hypertension is associated with an acceleration of the atherosclerotic process. The present data indicate that the increased stretch of the intimal-medial tissue system associated with the increased intervascular pressure is probably a relatively minor mechanism to explain the increased macromolecular flux into the intimal space suggesting that other mechanisms such as endothelial injury or increased convective force are probably more important.

Proposed Course: These studies are being extended to define more closely the nature of the aforementioned time course of altered arterial uptake associated with stretch and also to define more explicitly the aspects of the above observations that may be artifactually related to the in vitro methodology itself.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02806 06 EA

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
Blood velocity profiles and hemodynamic stresses in the aorta and its major branches

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

PI:	D.J. Patel	Medical Officer	EA NHLBI
Other:	H.B. Atabek	Professor, Catholic University of America	
	S.C. Ling	Professor, Catholic University of America	

COOPERATING UNITS (if any)
Mechanical and Civil Engineering Departments, Catholic University of America, Washington, DC

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION
Vascular Physiology Section

INSTITUTE AND LOCATION
NIH/NHLBI-DIR, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.6	.4	.2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The long-range purpose of this project has been to study the blood velocity profiles and hemodynamic stresses in the aorta and its major branches. The topic of present interest is to develop and test the nonlinear theory of pulse propagation in vivo in dogs. The theory will predict flow fields at more distal sites along the aorta from pressure gradient measurements made at a proximal site.

PHS-6040
(Rev. 10-76)

PROJECT DESCRIPTION

Objective: To test a new method of pulse propagation analysis in a model of an artery. This method can determine complete flow fields in an artery at distal sites using pressure and pressure-gradient measurements made at its proximal end and the pressure-radius relationship measured along the segment.

Methods: A specially designed model system was used to test the theory. The model was made of silicone rubber and consisted of a heart pump, a tapered section, a long uniform tube (the test section) followed by another tapered section and a long uniform tube of smaller diameter. A glycerine-water mixture was used as the test fluid. This system, with the help of electronic controls, could produce pulsed waves similar to those encountered in dogs. Pressure and centerline velocities were measured at the proximal and distal ends of a 20-cm long segment of the uniform tube, far away from its entrance. The data obtained at the proximal end were used as input for the computations. The data obtained at the distal end were used to check the predicted results.

Major Findings: Predicted values of the pressure, pressure-gradient, average velocity and centerline velocity waves at the distal end of the segment were close to the corresponding experimentally determined values. Numerical experiments indicated that the differences between the predicted and measured quantities were largely due to nonuniformity of the elastic properties in the test section of the tube.

Significance: Hemodynamic stresses are thought to play a central role in the etiology of early atherosclerosis. In order to study this role quantitatively, we need to measure, in vivo, flow fields in the critical areas of the circulatory system. The study described above represents a step toward this goal.

Proposed Course: After a few appropriate animal studies, this project will be terminated.

PUBLICATIONS

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 02807 06 EA

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
Vascular mechanics: local properties of the intimal layer of large arteries

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

PI: D.J. Patel — Medical Officer — EA NHLBI

Other: R.N. Vaishnav — Professor, Catholic University of America

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Civil Engineering Department, Catholic University of America, Washington, DC

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION
Vascular Physiology Section

INSTITUTE AND LOCATION
NIH/NHLBI-DIR, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.6	.4	.2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Local rheology of the vascular intima was studied in two ways. One, a new microindentation apparatus was designed and fabricated to map the local compliance of the intimal layer of the aorta. Two, the strength of the endothelial surface was studied by exposing it to a saline jet of known strength and quantifying the resulting damage to the surface. It is hoped that these studies will help in understanding the role of mechanical factors in atherogenesis.

PHS-6040
(Rev. 10-76)

PROJECT DESCRIPTION

Objective: To study local rheology of the endothelial surface and the intimal layer of large arteries with a view toward understanding its role in atherogenesis.

Methods: Basically two methods have been developed to study the rheology of the endothelial surface. 1) The microindentor technique to study the compliance of the intimal surface was developed earlier in our laboratory by Gow and Vaishnav. The prototype apparatus, however, was not rugged enough for routine mapping of the compliance of the intimal layer. A new, rugged and more stable instrument based on an air-bearing principle has now been built and is currently being used to study the intimal layer compliance. 2) A saline jet method to study the strength of the endothelial surface has also been developed in our laboratory. During the preceding years the technique to create and quantify lesions on the endothelial surface was developed. The current effort has been to calibrate the jet for the shear stress field it would create on the surface. This has been approached in two ways: first, by actual measurements of shear stress in a jet model 10 times the size of the prototype, and, second, using numerical techniques to compute the stress field.

Major Findings: 1) The new apparatus is being used to collect preliminary data on the compliance of the aortic surface. These will be correlated with Dr. Fry's permeability studies. 2) A typical lesion created by a jet on the endothelial surface of the thoracic aorta is annular in shape. From the knowledge of its size and the shear stress field, it is possible to estimate the shear strengths of the endothelium. Stresses in excess of 400 dyn/cm^2 seem to damage the endothelial cells, which is consistent with Fry's results.

Significance: Local rheological properties of the intimal layer which include the strength of the endothelial surface and the compliance of the intima are known to affect the permeability of the endothelial surface to various macromolecules. There is considerable evidence that the permeability of the vascular interface plays a significant role in atherogenesis.

Proposed Course: We hope to complete these two studies and terminate the project.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02810 08 EA

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
Hyperlipoproteinemia and atherosclerosis: changes in plasma lipoproteins and apolipoproteins induced by cholesterol feeding

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

PI:	R.W. Mahley	Head, Comparative Atherosclerosis and Arterial Metabolism Section	EA NHLBI
	K.W. Weisgraber	Expert	EA NHLBI
	D.L. Fry	Chief, Laboratory of Experimental Atherosclerosis	EA NHLBI
Other:	K.S. Holcombe	Chemist	EA NHLBI

COOPERATING UNITS (if any)
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LAB/BRANCH
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SECTION
Comparative Atherosclerosis and Arterial Metabolism Section

INSTITUTE AND LOCATION
NIH/NHLBI-DIR, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

This study will attempt to correlate plasma lipoprotein changes induced by cholesterol-rich diets with the development of accelerated atherosclerosis in various animal models. Characterization of cholesterol-induced lipoproteins in dogs, swine, monkeys, rats, and rabbits has shown that various species have similar metabolic responses to increased dietary cholesterol. The hyperlipoproteinemia has certain consistent features, including the occurrence of beta-very low density lipoproteins, an increase in low density lipoproteins, and the appearance of a unique lipoprotein, HDL_C. The occurrence in increased concentration of a specific apoprotein (the arginine-rich apoprotein, apo-E) with all these cholesterol-induced lipoproteins suggests an important role for this protein in cholesterol metabolism and possibly in accelerated heart disease. The various animal lipoproteins are being compared to purified human plasma lipoproteins.

PHS-6040
(Rev. 10-76)

PROJECT DESCRIPTION

Objectives: 1) To characterize the lipoproteins and apoproteins from control dogs, miniature swine, Patas monkeys, rats, and rabbits, and to compare these to changes induced by cholesterol feeding. 2) To correlate the type of hyperlipoproteinemia with the type, distribution, and degree of atherosclerosis. 3) To compare animal lipoproteins with those of man.

Methods: The various animal species, including dogs, swine, monkeys, rats, and rabbits, were fed diets containing 0.5 to 2.0% cholesterol, as described previously. Plasma lipoproteins were isolated by the combination of ultracentrifugation and Geon-Pevikon block electrophoresis. The purified lipoproteins were characterized with respect to electrophoretic mobility, immunochemical reactivity, size by electron microscopy, chemical composition, and apoprotein content. The apoproteins were isolated and purified by Sephadex and DEAE column chromatography. Analyses of the apoproteins included amino acid analysis, N- and C-terminal amino acids, and molecular weights.

Major Findings: Dogs, miniature swine, rats, rabbits, and Patas monkeys fed high-cholesterol diets had similar lipoprotein responses, associated with the development of atherosclerosis. Animals on a low-cholesterol diet served as controls. The characteristics of the hyperlipoproteinemia associated with atherosclerosis were as follows: 1) The B-VLDL become prominent lipoproteins. These are beta-migrating lipoproteins in the d<1.006 fraction which resemble the beta-VLDL of human Type III hyperlipoproteinemia, particularly with respect to the prominence of the "arginine-rich" apoprotein (apo-E). 2) LDL and the intermediate lipoproteins (IDL) are present in increased concentrations and are variably enriched in apo-E. These lipoproteins and the beta-VLDL may represent remnants of intestinal lipoproteins induced to transport the dietary lipids. 3) A unique class of lipoproteins, which we have called HDL_C, is a consistent feature following cholesterol feeding. These lipoproteins are cholesterol rich and contain the "arginine-rich" apoprotein and A-1. They lack the B-apoprotein. The HDL_C are important regulators of sterol synthesis in aortic smooth muscle cells and fibroblasts (see Z01 HL 02813 07 EA).

Characterization of the "arginine-rich" apoprotein suggests homology of this protein among the species. Apo-E from various species are similar by amino acid analysis and contain 12 moles% arginine. This apoprotein appears to play an essential role in cholesterol transport between lipoproteins and possibly between lipoproteins and the aortic wall (atherosclerotic lesion).

Significance: Characterization of cholesterol-induced hyperlipoproteinemia and development of animal models resembling human disease will enable us to better understand human lipoprotein metabolism. In addition, these studies are designed to correlate the type of hyperlipoproteinemia with the type, distribution, and degree of experimentally induced atherosclerosis.

Proposed Course: The project will continue along the lines indicated above with an extension in the detailed characterization of human lipoproteins.

PUBLICATIONS

1. Weisgraber, K.H., R.W. Mahley, G. Assmann. The rat arginine-rich apoprotein and its redistribution following injection of iodinated lipoproteins into normal and hypercholesterolemic rats. Atherosclerosis. 28: 121-140, 1977.

2. Tall, A.R., D. Atkinson, D.M. Small, R.W. Mahley. Characterization of the lipoproteins of atherosclerotic swine. J Biol Chem. 252: 7288-7293, 1977.

3. Mahley, R.W., K.H. Weisgraber, T.P. Bersot, T.L. Innerarity. Effects of cholesterol feeding on human and animal high density lipoproteins. In High Density Lipoproteins and Atherosclerosis, edited by A.M. Gotto, Jr., N.E. Miller, M.F. Oliver. Elsevier Press. Amsterdam. pp. 149-176, 1978.

4. Weisgraber, K.H., R.W. Mahley. Apoprotein (E--A-II) complex of human lipoproteins. I. Characterization of this mixed disulfide and its identification in a high density lipoprotein subfraction. J Biol Chem. 253: 6281-6288, 1978.

5. Innerarity, T.L., R.W. Mahley, K.H. Weisgraber, T.P. Bersot. Apoprotein (E—A-II) complex of human plasma lipoproteins. II. Receptor binding activity of a high density lipoprotein subfraction modulated by apo(E—A-II). J Biol Chem. 253: 6289-6295, 1978.

6. Atkinson, D., A.R. Tall, D.M. Small, R.W. Mahley. Structural organization of the lipoprotein HDL_C from atherosclerotic swine. Structural features relating the particle surface and core. Biochemistry. 17: 3930-3933, 1978.

7. Weisgraber, K.H., T.P. Bersot, R.W. Mahley. Isolation and characterization of an apoprotein from the d<1.006 lipoproteins of human and canine lymph homologous with the rat A-IV apoprotein. Biochem Biophys Res Commun. 85: 287-292, 1978.

8. Mahley, R.W. Dietary fat, cholesterol, and accelerated atherosclerosis. In Atherosclerosis Reviews, edited by R. Paoletti, A.M. Gotto, Jr. Raven Press. New York. Vol. 5, pp. 1-34, 1979.

9. Mahley, R.W., K.H. Weisgraber, T.L. Innerarity. Interaction of plasma lipoproteins containing the B and E apoproteins with heparin and cell surface receptors. Biochim Biophys Acta. In press.

10. Mahley, R.W., K.H. Weisgraber. Subfractionation of high density lipoproteins into two metabolically distinct subclasses by heparin affinity chromatography and Geon-Pevikon electrophoresis. NHLBI Monograph on High Density Lipoproteins. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02811 08 EA

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
Aortic metabolism of plasma lipoproteins

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

PI:	R.W. Mahley	Head, Comparative Atherosclerosis and Arterial Metabolism Section	EA NHLBI
	D.L. Fry	Chief, Laboratory of Experimental Atherosclerosis	EA NHLBI
	K.H. Weisgraber	Expert	EA NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION
Comparative Atherosclerosis and Arterial Metabolism Section

INSTITUTE AND LOCATION
NIH/NHLBI-DIR, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	.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)

This study seeks to determine which lipoproteins are transported across the aortic wall, how these lipoproteins are transported, and what their ultimate fate is in the endothelial tissue. The aorta can be maintained in vitro for several hours, using methods developed in the course of this project.

PHS-6040
(Rev. 10-76)

PROJECT DESCRIPTION

Objectives: To determine 1) which classes of plasma lipoproteins are involved in aortic transport; 2) whether these lipoproteins are transported as intact macromolecules or as components of surface-hydrolyzed molecules; and 3) the fate of the lipoprotein components metabolized by the aorta.

Methods: The in vitro transport method (described by Dr. D.L. Fry) is used to study aortic endothelial transport of the plasma lipoproteins under controlled conditions. Presently, the dog is being used as the experimental model, but we will soon extend this to the miniature swine. The components of canine plasma lipoproteins (VLDL, LDL, HDL_1 and HDL_2) are labeled with various radioisotopes. Initially, we attempted to use ^{125}I as our protein tag. After exhaustive studies we conclude that it is impossible to limit the ^{125}I to the protein moieties, and variable amounts of lipids are labeled. The ^{125}I lipid label is unstable. We are now able to label the protein moieties of the canine lipoproteins with ^{35}S-methionine in vivo. At the same time, we are able to label the phospholipids with ^{32}P-orthophosphate. ^{14}C-cholesterylesters and ^{3}H-free cholesterol moieties of these lipoproteins are labeled in vitro by the exchange method by Avigan. Uptake of label and the metabolism of the lipoproteins are followed by analysis of changes in the incubation media, examination of the aorta by direct isotope counting following oxygen combustion and by light and electron microscopic autoradiography.

Major Findings: Preliminary findings indicate the feasibility of this approach to the study of aortic endothelial transport and metabolism of plasma lipoprotein. Methodologic problems and validation of techniques continues to be a major component of this project. In addition to validation of the in vitro technique (described in a separate project report by Dr. D.L. Fry), methodology for the quantitation of four separate radioisotopes (^{14}C, ^{3}H, ^{35}S, and ^{32}P) has been established. The labeled plasma lipoproteins or a dried portion of the aorta following an in vitro transport study are placed in an oxygen combustion flask and ignited. ^{35}S-methionine which is the protein tag and ^{32}P which is the phospholipid tag are converted to inorganic sulfate and phosphate, respectively. These isotopes remain in the flask and are quantitated together by standard double label liquid scintillation counting. The flask is heated to drive off the tritium in the form of $^{3}H_2O$ and ^{14}C in the form of $^{14}CO_2$. The $^{14}CO_2$ is collected by bubbling the gas through a base converting it to an insoluble carbonate and the $^{3}H_2O$ is collected on a condenser in an ice bath. Isotope recovery is greater than 90% and modifications are being made to increase the efficiency of the method.

Significance: It is agreed by most that 1) cholesterol within atheromata is derived largely from plasma lipoproteins and that 2) plasma lipoproteins can be detected within the same lesions. However, the mode of transport and the quantitative significance of lipoproteins in the lesions are far from clear. Serious questions remain as to whether the lipoproteins cross the endothelial surface intact or whether they are hydrolyzed at the surface with only some of the components entering the tissue. Our in vitro approach to this problem should shed light on this most difficult problem of

atherosclerosis research as well as add to our knowledge of lipoprotein metabolism.

Proposed Course: This project will be continued along the lines indicated above. It will also be extended to the miniature swine and nonhuman primates for comparative studies.

PUBLICATIONS

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02812 10 EA

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
Animal models for study of atherosclerosis

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

PI:	R.W. Mahley	Head, Comparative Atherosclerosis and Arterial Metabolism Section	EA NHLBI
	D.L. Fry	Chief, Laboratory of Experimental Atherosclerosis	EA NHLBI
	G.W. Melchior	Investigator	EA NHLBI
Other:	J.E. Pierce	Chief, Section on Laboratory Animal Medicine and Surgery	OD NHLBI
	D.K. Johnson	Chief, Veterinary Medicine and Surgery Section	VR DRS
	R.M. Jaffe	Staff Physician	CP CC
	R.L. Killens	Chief, Comparative Medicine Unit	VR DRS
	T.L. Wolfle	Assistant Head, Carnivore Unit	VR DRS

COOPERATING UNITS (if any)
Veterinary Resources Branch, DRS; Clinical Pathology, CC: Comparative Medicine Unit, DRS; Meloy Laboratories, Springfield, VA

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION
Comparative Atherosclerosis and Arterial Metabolism Section

INSTITUTE AND LOCATION
NIH/NHLBI-DIR, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	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 study is to develop a dietary protocol and maintain metabolic conditions which are conducive to the development, in various animal species, of experimental atherosclerosis similar to human disease. This has been achieved in dogs, miniature swine, and Patas monkeys. For these species the source of dietary fat has been found to profoundly affect the type, distribution, and severity of the disease. Atherosclerosis produced by diets containing beef tallow is severe and is associated with gross arterial thrombosis and occlusive vascular disease.

PHS-6040
(Rev. 10-76)

PROJECT DESCRIPTION

Objective: To determine the suitability of a variety of animals as models for studying human atherosclerosis.

Methods: The animal models which have been studied in varying detail are Patas monkeys, miniature swine, dogs, rabbits, and rats. The experimental conditions under which the pathologic processes in these animals can be made to resemble those in man have been detailed in previous project reports. Briefly, the disease can be induced in rabbits, swine, and monkeys by feeding diets high in cholesterol and fat (lard, beef tallow), whereas the disease can be induced in dogs and rats to a comparable extent only if hypothyroidism is also induced. In dogs, the dietary fats have included cottonseed oil, pork lard, beef tallow, safflower oil, and peanut oil.

Blood chemistries, including detailed lipoprotein studies, are monitored during the experimental period. Detailed lipid analyses of the platelets and erythrocytes from the animals on various diets are done by standard procedures. At termination each animal is examined in detail using the standardized necropsy procedures described previously. Topographic distribution and histologic characteristics of aortic, coronary, and peripheral arteries are compared to those of human atherosclerosis. The comparative human material is derived from young adults dying traumatic deaths, unselected hospital cases, and patients with documented types of hyperlipoproteinemia. A computerized coordinate system for plotting the topographic distribution of the atherosclerotic lesions has been developed.

Major Findings: The animal colony studies in progress for several years continue to provide the basis for our understanding of several key questions in the field of lipoprotein metabolism and atherosclerosis. One of the strengths of our program has been the ability to compare the response of atherosclerosis-resistant (dog and rat) species with susceptible (swine and monkey) species and to relate these findings to human disease. The approach has provided fundamental, basic information in several areas as follows:

1. Lipoprotein Metabolism. Certain consistent features of cholesterol-induced hyperlipoproteinemia in the various species have been described including the occurrence of a previously unrecognized class of plasma lipoproteins. The detailed understanding of the types of changes induced by cholesterol feeding in the animals (dogs, swine, rats, rabbits, and monkeys) has provided a background for studying the changes which occur with cholesterol feeding in man. A fundamental observation which is being actively studied in our laboratory is that the consumption of a cholesterol-rich diet by man, with or without an elevation of plasma cholesterol, alters the plasma lipoproteins in ways which resemble the changes observed in the lower species. In the animal studies such changes are associated with accelerated atherosclerosis.

2. Regulation of Cholesterol Metabolism in Arterial Smooth Muscle Cells and Fibroblasts by Plasma Lipoproteins. A recent contribution of our laboratory, which was provided by the uniqueness of the cholesterol-induced lipoproteins

of dogs and swine, has been the identification of the determinants responsible for lipoprotein binding to specific cell surface receptors. It is now established that the protein moieties of the lipoproteins are responsible for specific binding, that the B and arginine-rich (E) apoproteins are responsible for the receptor binding, and that arginyl residues of the proteins are functionally important in the lipoprotein recognition site. In addition, the regulation of the rate and extent of intracellular deposition of cholesteryl esters by arterial cells appears to be controlled by the type of lipoprotein which interacts with the receptor (see Z01 HL 02817 03 EA).

3. Role of Dietary Fat in Determining the Severity of Atherosclerosis and the Associated Complications of Thromboatherosclerosis. Dogs fed high-cholesterol diets containing either saturated or unsaturated fats develop two types of atherosclerosis, different in both distribution and severity. Diets containing saturated fat cause more severe atherosclerosis and a significant increase in the occurrence of thrombosis and the complications of thrombo-atherosclerosis (visceral organ infarction). Thromboatherosclerosis in dogs (and more recently in swine) on a high saturated fat-cholesterol diet has been correlated with platelet hypersensitivity to aggregation and release. These models provide for the first time an experimental approach by which to determine whether the role of the platelet in atherosclerosis is primary or secondary. Lipid analyses have revealed significant changes in the fatty acid composition of the platelets depending upon the type of diet. In addition, these well-characterized models provide the opportunity to determine experimentally the factors which alter the course of the disease process. The animal models provide the only approach to many of the questions which need to be explored if we are to understand the role of diet, trace elements and vitamins, plasma lipoprotein levels, and hemostatic function in the pathogenesis of atherosclerosis. Many of the advances we have made relate directly to observations made first in the animals and then extended to man.

Significance: The "atherosclerotic process" is, in fact, an ensemble of processes occurring at the cellular, physicochemical, biochemical, and biophysical level in the arterial intima. The purpose of this program is to identify as many of these fundamental processes as possible, establish which are relevant to the disease process in man, and study these in great detail in the animal model, wherein the pertinent variables can be measured or controlled with a rigor not possible in man.

A clear definition of the role of diet in the development of atherosclerosis in man is of utmost importance.

Proposed Course: The pursuit of the above objectives will continue with our NIH collaboration.

PUBLICATIONS

1. Mahley, R.W., T.L. Innerarity, K.H. Weisgraber, D.L. Fry. Accumulation of lipid by aortic medial cells in vivo and in vitro. Am J Pathol. 87: 205-226, 1977.

2. Pitas, R.E., G.J. Nelson, R.M. Jaffe, R.W. Mahley. Δ15,18-Tetracosadienoic acid content of sphingolipids from platelets and erythrocytes of animals fed diets high in saturated or polyunsaturated fats. Lipids. 13: 551-556, 1978.

3. Pitas, R.E., G.J. Nelson, R.M. Jaffe, R.W. Mahley. Effects of diets high in saturated fat and cholesterol on the lipid composition of canine platelets. Lipids. 14: 469-477, 1979.

4. Mahley, R.W. Dietary fat, cholesterol, and accelerated atherosclerosis. In Atherosclerosis Reviews, edited by R. Paoletti, A.M. Gotto, Jr., Raven Press. New York. Vol 5, 1-34, 1979.

5. Reitman, J.S., R.W. Mahley. Yucatan miniature swine lipoproteins: changes induced by cholesterol feeding. Biochim Biophys Acta. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02813 07

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
Tissue culture studies of aortic smooth muscle cells and skin fibroblasts: cell growth and metabolism in response to incubation with various lipoprotein classes

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

PI:	T.P. Bersot	Senior Investigator	EA NHLBI
Other:	R.W. Mahley	Head, Comparative Atherosclerosis and Arterial Metabolism Section	EA NHLBI
	D.L. Fry	Chief, Laboratory of Experimental Atherosclerosis	EA NHLBI

COOPERATING UNITS (if any)
Meloy Laboratories, Springfield, VA

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION
Comparative Atherosclerosis and Arterial Metabolism Section

INSTITUTE AND LOCATION
NIH/NHLBI-DIR, Bethesda, MD

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)

These studies focused on the mechanism by which plasma lipoproteins deliver lipids to cultured cells. Using chylomicrons and Lp(a) we have shown that the presence of apolipoproteins B and E in these lipoproteins does not assure binding to the high affinity receptor. Partial delipidation of these lipoproteins renders them capable of binding and suggested that the interaction of these apolipoproteins with lipid is important in the binding process.

520

PHS-6040
(Rev. 10-76)

PROJECT DESCRIPTION

Objectives: 1) To study the effect of various lipoprotein classes upon aortic smooth muscle cell proliferation. 2) To study the effects of various lipoprotein classes upon smooth muscle cell and skin fibroblasts cholesterol metabolism. 3) To establish strains of swine and canine endothelial cells in culture.

Methods: 1) Standard techniques were used in determining cell proliferation in response to various classes of lipoproteins. 2) Electron microscopy was used to establish that cells were similar to smooth muscle and endothelial cells as reported in the literature. 3) Cellular cholesterol synthesis was assessed by measuring the incorporation of [I-^{14}C] acetate into cholesterol and cholesteryl esters. 4) Lipoproteins were partially delipidated with ether by previously described methods. 5) Binding, uptake, and degradation of iodinated lipoproteins were measured by standard techniques.

Major Findings: 1) The ability of lipoproteins to be bound, internalized, and degraded by normal fibroblasts was previously found to be dependent upon the presence of apoproteins B and E. 2) The configuration of these apoproteins in the lipoprotein is also important because neither freshly isolated chylomicrons nor Lp(a) were found to bind to the high affinity receptor despite the presence of apoproteins B and E in both lipoproteins. Partial delipidation of these two lipoproteins resulted in binding of the partially delipidated particles.

Significance: The finding of lipoproteins which do not bind to the high affinity receptor despite the presence of apoproteins B and E suggests that other factors than these apoproteins are important in the recognition and uptake of lipoproteins by cells.

Proposed Course: This project has been completed.

PUBLICATIONS

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02814 04 EA

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
Topographic analyses of endothelial surface permeability and atherosclerosis

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

PI:	D.L. Fry	Chief, Laboratory of Experimental Atherosclerosis	EA NHLBI
Other:	R.W. Mahley	Head, Comparative Atherosclerosis and Arterial Metabolism Section	EA NHLBI
	D.K. Johnson	Chief, Veterinary Medicine and and Surgery Section	VR DRS
	J.E. Pierce	Chief, Section on Laboratory Animal Medicine and Surgery	OD NHLBI
	J.M. DeLeo	Computer Systems Analyst	CR CSL
	E. Hall	Electronics Engineer	BEI DRS

COOPERATING UNITS (if any)
Veterinary Resources Branch, DRS; Computer Systems Laboratory, DCRT; Biomedical Engineering and Instrumentation Branch, DRS

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION
Vascular Physiology

INSTITUTE AND LOCATION
NIH/NHLBI-DIR, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.50	.25	1.25

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

To develop standardized necropsy and formating procedures to study topographic correlations in experimental atherosclerosis.

502

PROJECT DESCRIPTION

Objective: To develop standardized necropsy and formating procedures to study topographic correlations in experimental atherosclerosis.

Methods: A systematized necropsy procedure and a standardized format was developed for arranging and fastening the opened arterial tree to reflective panels for optical scanning and analysis. This standardized format makes possible quantitative analysis of the topographic distribution of intimal Evans blue dye accumulation (a measure of the permeability of the wall to plasma substances) and of intimal lipid deposition along the arterial tree of experimental animals. This standardized format may be expressed in a normalized coordinate system for automatic optical scanning and computer pattern analysis or may be used directly for comparative visual analysis. This format permits careful, detailed comparisons of areas of increased arterial wall permeability with areas of intimal lipid deposition at identical sites from animal to animal.

Major Findings: Application of these techniques to a recently completed study in experimental atherosclerosis in swine demonstrated that the disease pattern occurs with an extraordinary congruency from animal to animal. It was also shown that the patterns of increased endothelial permeability in normal animals are very similar to the patterns of intimal lipid deposition that occur in animals on an atherogenic regimen. At certain local sites, that were characterized by very high permeability in normal animals, a characteristic atheromatous lesion occurred which, under the above standardized necropsy procedures, appeared as a tri-colored lesion. These lesions were characterized by relatively normal surrounding intimal surface (white), a highly permeable (blue) center bordered by an annular but otherwise typical atheromatous plaque (red). With time, these lesions were shown to form mature atheromatous plaques in the abdominal aorta; however, in the thoracic aorta and subclavian artery these appear to mature more slowly.

Significance: The extraordinary congruency of the disease patterns from animal to animal in a given species, as well as to the patterns of permeability in the normal animal of that species, represents a major finding of fundamental importance not only in linking the role of increased permeability to the subsequent development of this disease but also in permitting one to develop new strategies in experimental design to gain deeper insight into the associated atherogenic processes. The predictability of this process at given locations permits one to design studies to follow the sequence of events leading from the "earliest lesion" on through the complicated lethal atheromatous plaque. This capability was heretofore unavailable for lack of a standardized quantitative approach to the analyses of the disease topography and permeability topography. Moreover, the occurrence of the tri-color lesion provides the opportunity to study an apparent spectrum of atherogenic processes in a very discrete anatomical region.

This methodology will make possible an approach to a number of fundamental questions: Why do some regions of moderate endothelial permeability progress only to the fatty streak lesions whereas others go on to mature atherosclerotic lesions? Why do certain local regions of extraordinarily high permeability appear to be relatively immune to lipid deposition? What is the sequence of events leading from a fatty streak to a lethal atheromatous plaque? How are the patterns of streaking and plaquing influenced by hemodynamic alterations, hematologic manipulations, dietary triglycerides, and exercise?

Proposed Course: The progress of this large project has been slowed by several unanticipated problems related to the large data processing problems posed by this objective. Analysis has involved relatively detailed and sophisticated computer data processing techniques. We have finished the necessary pilot studies to establish the methodological and computational concepts. These are now being implemented with the necessary image capture and processing hardware and software. Initial applications will be to assess the influence of a variety of dietary and other factors on the evolution of this important disease process.

PUBLICATIONS

1. Lutz, R.J., J.N. Cannon, K.B. Bischoff, R.L. Dedrick, R.K. Stiles, D.L. Fry. Shear stress patterns in a model canine artery: Their relationship to atherosclerosis. In Quantitative Cardiovascular Studies. Clinical and Research Applications of Engineering Principles, edited by N.H.C. Hwang, D.R. Gross, D.J. Patel. University Park Press. Baltimore. pp. 233-237, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02815 03 EA

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
A quantitative autoradiographic method for the measurement of radiolabeled protein distribution across arterial tissue

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

PI:	D.L. Fry	Chief, Laboratory of Experimental Atherosclerosis	EA NHLBI
Other:	R.W. Mahley	Head, Comparative Atherosclerosis and Arterial Metabolism Section	EA NHLBI
	S.J. Lewis	Histologist	EA NHLBI
	F. Plowman	Mechanical Engineer	EA NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION
Vascular Physiology Section

INSTITUTE AND LOCATION
NIH/NHLBI-DIR, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.80	.30	1.50

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)

A quantitative autoradiographic technique is being developed using electron probe microanalysis to determine the developed silver distribution across the autoradiograph. It appears that the local tissue concentration of radiolabeled protein is linearly related to the silver signal from the electron probe system.

505

PHS-6040
(Rev. 10-76)

PROJECT DESCRIPTION

Objectives: To develop methodology for study of plasma protein concentration distributions across arterial tissue systems using electron probe x-ray microanalysis to quantify autoradiographic silver across histologic preparations.

Methods: Arterial specimens were placed in a specially designed device which permitted exposure of the intimal surface of the arterial tissue to radiolabeled albumin under controlled experimental conditions. Selected exposure periods and concentrations of radiolabeled albumin were chosen to vary the uptake of radiolabeled protein by the tissue at various sites along the vessel. In one set of experiments, uptakes were varied from zero to levels of activity in excess of those to be encountered in any experimental application of this technique. In the other set of experiments, paired wells were chosen such that adjacent sites would have the same tissue concentration of radioactivity and thus the distribution of activity at each site could be compared by independent techniques. By one technique, the radioactivity in 15 μm en face serial microtomy sections taken across the wall was measured directly. The other technique was quantitative autoradiography which was done on a matched tissue specimen. Following photographic development, the distribution of silver induced by the underlying radioactivity was quantified by electron probe microanalysis.

Major Findings: The volume-averaged signal from the electron microprobe analysis of the silver was found to be directly proportional to the volume-averaged tissue concentration of radiolabeled protein from zero to a range in excess of that expected in any application of this technique. Moreover, the comparison of the distribution of the silver across the autoradiograph was congruent with the distribution of radioactivity as determined by the serial microtomy sections.

Significance: This new methodology makes it possible to determine with relatively high resolution (±10 μm) the concentration of radiolabeled proteins across fixed tissue. Although this technique should have broad application to studies of radiolabeled protein distributions across other tissues, its major significance in the present work is that it allows one to measure one of the principal variables in transvascular transport processes, i.e., the concentration gradients that drive the macromolecular flux. This will permit study of local transport processes in arterial tissue and their role in atherogenesis.

Proposed Course: This methodology is being applied to studies of transvascular macromolecular transport in relation to atherogenesis.

PUBLICATIONS

1. Brown, B.G., D.L. Fry. The fate and fibrogenic potential of subintimal implants of crystalline lipid in the canine aorta. Quantitative histological and autoradiographic studies. Circ Res. 43(2) 261-273, 1978.

2. Lutz, R.J., J.N. Cannon, K.B. Bischoff, R.L. Dedrick, R.K. Stiles, D.L. Fry. Shear stress patterns in a model canine artery: Their relationship to atherosclerosis. In Quantitative Cardiovascular Studies. Clinical and Research Applications of Engineering Principles, edited by N.H.C. Hwang, D.R. Gross, D.J. Patel. University Park Press. Baltimore. pp. 233-237, 1979.

3. D.L. Fry, R.N. Vaishnav. Mass transport in the arterial wall. In Basic Hemodynamics (and Its Role in Disease Processes). University Park Press. Baltimore. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02816 03 EA

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
The combined convective and diffusive transport of Evans blue dye-albumin into the deendothelialized aortic wall

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

PI: D.L. Fry	Chief, Laboratory of Experimental Atherosclerosis	EA NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION
Vascular Physiology Section

INSTITUTE AND LOCATION
NIH/NHLBI-DIR, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.50	.25	1.25

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Macromolecular transport into the arterial wall occurs by diffusive as well as convective processes. The present studies, for the first time, quantified the relative order of magnitude of diffusive forces as compared to convective forces in transvascular transport across the deendothelialized aortic wall.

508

PHS-6040
(Rev. 10-76)

PROJECT DESCRIPTION

Objective: To study the processes of transarterial macromolecular transport using the albumin-Evans blue dye ligand system.

Methods: Rapidly excised descending thoracic aortas from dogs were placed in a specially designed multichambered device in which the chemical milieu on the intimal surface, as well as the transmural pressure, could be under experimental control. The endothelial surface was discretely removed with a wet camel hair brush. Albumin was labeled with EBD and placed in the chambers on the intimal surface of the vessel. The well chambers along the aorta were divided into sets of three. One well in each set was maintained at zero transmural pressure, one well at the positive transmural pressure, and the third at a negative transmural pressure of 100 mm Hg pressure. Four sets of wells along the artery allowed four different exposure times to be studied such that three different curves could be constructed, each with four exposure time points on it representing the corresponding uptake of EBD-albumin. One curve represented the uptake with 100 mm Hg positive pressure, one with 100 mm Hg negative pressure, and one with zero transmural pressure.

Major Findings: Comparison of the ordinate differences on these three curves allowed calculation of the increment of uptake induced by the pressure as compared to the uptake of purely diffusive forces. Comparison of the uptake curve from the positive transmural pressure to that for the negative transmural pressure allowed calculation of the increment increased influx of stretch alone. The pressure driven flux into the wall was found (at physiologic levels of pressure of 100 mm Hg) to be of the same order as that for the steady-state diffusive flux into the wall. Thus, in considering transport into the deendothelialized wall, both driving forces, diffusive and convective, must be taken into account; and, moreover, under conditions of hypertension, it is likely that the pressure driven or convective flux may become the predominant factor in areas of endothelial injury.

Significance: A large body of evidence both from this laboratory, as well as from others, indicates that the atherogenic precursors probably come from the blood phase into the wall as various complexes of proteins. Moreover, the cholesterol-rich remnants from metabolism of these lipoprotein substances are probably carried out of the wall by other protein or detergent-like substances which are also transported through the wall. While albumin itself can carry both fatty acids and some cholesterol, its transport is of major interest to us as a prototype model for macromolecular transport and binding in the intimal space. Thus, while of inherent interest in albumin and fatty acid transport, the above studies begin to place, for the first time, macromolecular transport in vascular tissue on a physical-chemical footing that can be described unequivocally in terms of mathematical models. With respect to atherogenesis, the implications of diffusive transport are quite different from those of convective transport. As the relative importance of these transport mechanisms come into clearer perspective, it should be possible to gain deeper insights into a number of the puzzles presented by this disease process; why do fatty streaks occur at particular

sites along the aorta; what is the reason for the curious propensity of certain sites to go on to develop the lethal atheromatous plaque; what causes the imbalance between the transport of atherogenic precursors into the wall and the transport of the cholesterol-rich end products out of the wall.

Proposed Course: A variety of simple models have been examined in search of a single mathematical scheme to explain the observed transport behavior of the system. None of the simpler models, for which there are analytic solutions, appear to be adequate. Current efforts are directed toward evaluation of digital and analog computer methods of numerical analysis. The experiments are being repeated with radiolabeled albumin and will be extended to muscular as well as elastic arteries. New microdensitometric techniques are being developed so that transvascular concentration distributions may be examined simultaneously with corresponding uptake data.

PUBLICATIONS

1. Brown, B.G., D.L. Fry. The fate and fibrogenic potential of subintimal implants of crystalline lipid in the canine aorta. Quantitative histological and autoradiographic studies. Circ Res. 43(2) 261-273, 1978.

2. Lutz, R.J., J.N. Cannon, K.B. Bischoff, R.L. Dedrick, R.K. Stiles, D.L. Fry. Shear stress patterns in a model canine artery: Their relationship to atherosclerosis. In Quantitative Cardiovascular Studies. Clinical and Research Applications of Engineering Principles, edited by N.H.C. Hwang, D.R. Gross, D.J. Patel. University Park Press. Baltimore. pp. 233-237, 1979.

3. D.L. Fry, R.N. Vaishnav. Mass transport in the arterial wall. In Basic Hemodynamics (and Its Role in Disease Processes). University Park Press. Baltimore. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02817 03 EA

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
Protein modification and apoprotein characterization of plasma lipoproteins

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

PI:	R.W. Mahley	Head, Comparative Atherosclerosis and Arterial Metabolism Section	EA NHLBI
	K.H. Weisgraber	Expert	EA NHLBI

COOPERATING UNITS (if any)
Meloy Laboratories, Springfield, VA

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION
Comparative Atherosclerosis and Arterial Metabolism Section

INSTITUTE AND LOCATION
NIH/NHLBI-DIR, Bethesda, MD

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)

This project will entail the selective modification of certain amino acid residues of plasma lipoproteins, with a view toward determining the effects of such modifications on the interaction of the lipoproteins with surface receptors on cultured cells.

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PROJECT DESCRIPTION

Objectives: 1) To modify plasma lipoproteins with various protein reagents which react with specific amino acid residues, to study the effect of these modifications on the physical and chemical properties of the lipoproteins, and to determine whether these modifications alter cell surface receptor binding and/or degradation by fibroblasts and smooth muscle cells; 2) To isolate and characterize apoproteins from the plasma lipoproteins of man and of lower species and to establish homologies and differences among these apoproteins among the various species.

Methods: Several chemical reagents, when allowed to react with proteins, selectively react with specific amino acid residues. These reagents have been used successfully to modify numerous enzymes in an attempt to determine which amino acids are at or in the proximity of the "active site." We have reversibly modified the arginyl and lysyl residues of the apoproteins of LDL and HDL_c using cyclohexanedione and diketene, respectively. The extent and kinetics of the arginine and lysine modifications were determined by amino acid analysis. In addition, the effect of the modification on the binding, internalization, and degradation of the lipoproteins by cells in tissue culture was studied. A list of reagents which have been and will be employed and the respective amino acid residues with which they react includes: cyclohexanedione (CHD), arginine; N-acetylimidazole, lysine; diketene, lysine; B-mercaptoethanol, cysteine; N-ethylmaleimide, cysteine; iodoacetamide, cysteine; mercuribenzoate, cysteine; bromophenacyl bromide, histidine; and diamide, oxidation of sulfhydryl groups.

The plasma lipoprotein apoproteins were isolated by Sephadex and DEAE column chromatography and by preparative SDS gel electrophoresis. Characterization of the purified apoproteins included amino acid analysis, immunochemical properties, and molecular weight determination.

Major Findings: 1) We have found that selective modification of a limited number of arginyl and lysyl residues of human LDL and canine LDL or HDL_c abolishes the high-affinity binding of these lipoproteins to normal human fibroblasts. Amino acid analysis of the CHD-modified lipoproteins showed that approximately half of the arginyl residues, but no other amino acid residues, were involved. Diketene modification of less than 20% of the lysine residues abolished binding activity. The physical and chemical properties of the lipoproteins, including lipid composition, were unchanged. After removal of the CHD and diketene, the binding capacity of the lipoproteins was restored. These results demonstrated that the high-affinity binding specificity of LDL and HDL_c is localized to the apoprotein moiety and that specific arginines and lysines are directly involved or in close proximity to the lipoprotein recognition site. The effects of chemical modification on in vivo metabolism of plasma lipoproteins is being investigated. 2) We have isolated an apoprotein with a molecular weight of 46,000 from the $d<1.006$ fraction of patients with Type III hyperlipoproteinemia and from a subfraction of normal human $d=1.063-1.125$ (HDL_2) obtained by block electrophoresis. Treatment of

this apoprotein with disulfide reducing agents resulted in the formation of two subunits (37,000 and 8,500 MW). The 37,000 MW component co-electrophoresed with the arginine-rich apoprotein (apo-E), reacted with immunochemical identity, and had an amino acid composition indicative of apo-E. The 8,500 MW subunit has been identified as the A-II apoprotein. This 46,000 MW has been called the apo (E—A-II)complex. In tissue culture, the subfraction of HDL_2 which contained the (E—A-II)complex and A-I as major apoproteins (lacking apo-B) was relatively incapable of displacing ^{125}I-LDL from cell surface receptors of fibroblasts and behaved like HDL_3 (d=1.125-1.21). However, after reduction with conversion of apo (E—A-II) to apo-E, this lipoprotein competitively displaced ^{125}I-LDL (50% displacement of 25 μg of protein) and was bound, internalized, and degraded similar to LDL. Reduction of LDL and HDL_3 did not enhance their binding activity. The apo (E—A-II) may represent a functionally inactive form of the apo-E and may modulate binding. 3) We have isolated rat apo-E from various rat lipoproteins (VLDL, HDL , HDLc, and HDL) and established the identity of the apo-E from these various lipoproteins, as well as homology with human, swine, canine, and Patas monkey apo E, by amino acid analysis, co-electrophoresis, and immunochemistry. The apo-E was isolated by gel filtration, DEAE chromatography, and elution from SDS gels. Purified apo-E samples exhibited heterogeneity in their elution profiles on DEAE chromatography and showed multiple bands on Tris-urea gel electrophoresis. These multiple DEAE peaks were shown to be identical by amino acid and immunochemical analyses. However, differences in the sialic acid contents of the peaks were detected. These sialic differences appear to be responsible in part for the apo-E heterogeneity. Further characterization of apo-E heterogeneity in various species is under investigation.

Significance: The determination of the apoprotein content of various lipoproteins and the correlation of apoprotein content with metabolic behavior are important to our understanding of the lipoproteins and their role in cardiovascular heart disease. The selective modification of amino acid residues of specific lipoproteins and the effects of such modification on the binding, internalization, and degradation of these lipoproteins provides insight into the factors which are involved in the metabolism of lipoproteins by cells in culture. Cellular metabolism may be an important regulatory mechanism of the in vivo levels of the various lipoproteins.

The specific protein constituents (apoproteins) of the various classes of plasma lipoproteins appear to regulate the metabolism of the lipoproteins and to determine the fate of each class. Identification of these constituent proteins and their characterization are essential to the understanding of lipoprotein metabolism. The importance of specific apoproteins (B and E apoproteins, in particular) in the regulation of cellular cholesterol metabolism in fibroblasts and arterial smooth muscle cells has been established. Identification of the precise physicochemical properties responsible for the interaction of certain lipoproteins, and not others, with cell surface receptors may provide insight into the regulatory mechanisms of the deposition of cholesterol in the arterial wall.

Proposed Course: The project will continue along the lines indicated above.

PUBLICATIONS

1. Mahley, R.W., T.L. Innerarity, R.E. Pitas, K.H. Weisgraber, J.H. Brown, E. Gross. Inhibition of lipoprotein binding to cell surface receptors of fibroblasts following selective modification of arginyl residues in arginine-rich and B apoproteins. J Biol Chem. 252: 7279-7287, 1977.

2. Mahley, R.W., T.L. Innerarity. Interaction of canine and swine lipoproteins with the low density lipoprotein receptor of fibroblasts as correlated with heparin-manganese precipitability. J Biol Chem. 252: 3980-3986, 1977.

3. Mahley, R.W., T.L. Innerarity. Heparin-manganese precipitability of canine lipoproteins as correlated with binding to the cell surface receptors on fibroblasts. In Atherosclerosis IV, edited by G. Schettler, Y. Gotto, Y. Hata, G. Klose. Springer-Verlag. Berlin, pp. 55-56, 1977.

4. Mahley, R.W., T.L. Innerarity. Properties of lipoproteins responsible for high affinity binding to cell surface receptors of fibroblasts and smooth muscle cells. In Drugs, Lipid Metabolism, and Atherosclerosis, edited by D. Kritchevsky, R. Paoletti, W.L. Holmes. Plenum Press, pp. 99-127, 1978.

5. Innerarity, T.L., R.W. Mahley. Enhanced binding of cultured human fibroblasts of apo-E-containing lipoproteins as compared with low density lipoproteins. Biochemistry. 17: 1440-1447, 1978.

6. Weisgraber, K.H., T.L. Innerarity, R.W. Mahley. Role of the lysine residues of plasma lipoproteins in high affinity binding to cell surface receptors on human fibroblasts. J Biol Chem. 253: 9053-9062, 1978.

7. Mahley, R.W., K.H. Weisgraber, T.L. Innerarity, H.G. Windmueller. Accelerated clearance of low-density and high-density lipoproteins and retarded clearance of E apoprotein-containing lipoproteins from the plasma of rats after modification of lysine residues. Proc Natl Acad Sci. USA. 76: 1746-1750, 1979.

8. Pitas, R.E., T.L. Innerarity, K.S. Arnold, R.W. Mahley. Rate and equilibrium constants for binding of apo-E HDL_C (a cholesterol-induced lipoprotein) and low density lipoproteins to human fibroblasts: evidence for multiple receptor binding of apo-E HDL_C. Proc Natl Acad Sci. USA. 76: 2311-2315, 1979.

9. Innerarity, T.L., R.E. Pitas, R.W. Mahley. Binding of arginine-rich (E) apoprotein after recombination with phospholipid vesicles to the low density lipoprotein receptors of fibroblasts. J Biol Chem 254: 4186-4190, 1979.

10. Mahley, R.W., T.L. Innerarity, K.H. Weisgraber, S.Y. Oh. Altered metabolism (in vivo and in vitro) of plasma lipoproteins after selective chemical modification of lysine residues of the apoproteins. J Clin Invest. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02818 03 EA

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
The role in hyperlipoproteinemia of a high-density lipoprotein induced by cholesterol feeding

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

PI:	T.P. Bersot	Senior Investigator	EA NHLBI
Other:	R.W. Mahley	Head, Comparative Atherosclerosis and Arterial Metabolism Section	EA NHLBI
	D.L. Fry	Chief, Laboratory of Experimental Atherosclerosis	EA NHLBI

COOPERATING UNITS (if any)
Molecular Disease Branch - Clinical Service

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION
Comparative Atherosclerosis and Arterial Metabolism Section

INSTITUTE AND LOCATION
NIH/NHLBI-DIR, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Inpatient normal volunteers ate 6 eggs daily while taking diets rich in saturated or unsaturated fat. The plasma cholesterol concentrations increased transiently with both types of fat, but the greatest increase occurred with saturated fat. In the high-density lipoproteins of all subjects, a subfraction developed an increased content of apolipoprotein E. The type of dietary fat did not appear to influence the amount of this apo-E containing HDL. The apo-E HDL was able to bind to cells in tissue culture and compete with low-density lipoproteins.

PHS-6040
(Rev. 10-76)

PROJECT DESCRIPTION

Objectives: 1) To study the effects of eating cholesterol upon plasma lipoprotein metabolism. 2) To determine if eating cholesterol has any effect upon the accumulation of cholesterol in cells.

Methods: 1) Inpatient normal volunteers were fed baseline diets containing unsaturated fat and <300 mg cholesterol. Following stabilization of the plasma cholesterol concentration, 6 eggs per day were added to the baseline diet and again the plasma cholesterol concentration was allowed to stabilize. Next the dietary fat was changed from unsaturated to saturated fat, and the study was ended after the plasma cholesterol concentration had become stable. 2) Plasma lipid concentrations were measured and at the end of the baseline period and the two periods of egg-feeding, plasmaphereses were done. 3) Plasma lipoprotein subfractions were isolated and characterized by standard techniques. 4) Isolated lipoprotein fractions were tested in all culture experiments for their ability to bind to fibroblasts and influence cellular sterol metabolism.

Major Findings: As in previous studies, cholesterol feeding had little effect upon total plasma cholesterol concentrations once stabilization of the cholesterol concentration occurred. Cholesterol feeding, regardless of the type of dietary fat, increased the content of apolipoprotein E in a specific subfraction of HDL_2. This HDL_2 subfraction then became able to compete with low density lipoproteins for binding sites on the surface of fibroblasts.

Significance: The major significance of these studies was that eating cholesterol induced the production of an HDL which caused cholesterol to be transferred to cells. Thus, eating cholesterol may not increase plasma cholesterol concentrations but may still induce the deposition of cholesterol in tissue. It also appeared that the type of dietary fat had little effect upon the binding of apo-E containing HDL_2 to fibroblasts.

Proposed Course: This project will be completed within a few months.

PUBLICATIONS

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02819 03 EA

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

Plasma exchange in homozygous familial hypercholesterolemics

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

PI:	T.P. Bersot	Senior Investigator	EA NHLBI
Other:	H.B. Brewer	Chief, Molecular Disease Branch	MD NHLBI
	E. Schaefer	Staff Associate	MD NHLBI
	R.I. Levy	Director	NHLBI
	A.B. Deisseroth	Head, Experimental Hematology Section	C PO

COOPERATING UNITS (if any)

Molecular Disease Branch - Clinical Service
Pediatric Oncology Branch, NCI

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION
Comparative Atherosclerosis and Arterial Metabolism Section

INSTITUTE AND LOCATION
NIH/NHLBI-DIR, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.75	.25	.50

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Patients with homozygous familial hypercholesterolemia (HFH) have been treated by monthly plasma exchange to lower plasma cholesterol concentrations. One patient who was treated for 22 months had resolution of xanthomas but died during cardiac surgery. Plasma exchange was abandoned in the second patient, a retarded 17 year old girl, because of her refusal to take medications or follow dietary advice. The third patient, a 38 year old woman, had marked resolution of cutaneous xanthomas and improvement in her exercise tolerance.

518

PROJECT DESCRIPTION

Objectives: To assess the efficiency of chronic plasma exchange as a therapeutic tool to lower plasma cholesterol concentrations and reduce the tissue burden of cholesterol in HFH patients. 2) To study the effects of plasma exchange upon plasma lipoproteins.

Methods: Plasma exchanges were performed using a continuous flow centrifuge into which the anticoagulated patient's blood flows. The patient's plasma was collected and replaced with a plasma protein containing no lipoprotein cholesterol.

Major Findings: The regression of xanthomas in two patients treated was the first demonstration that this procedure could reduce tissue cholesterol burdens. Side effects have included two episodes of urticaria in one patient and a single ischemic episode in another.

Significance: Patients with HFA are resistant to drugs used to lower plasma cholesterol at present. They have a poor prognosis as a result of this. Plasma exchange offers an opportunity to reduce tissue cholesterol burdens and to determine if vascular atheromata will regress.

Proposed Course: This project has been completed.

PUBLICATIONS

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02820 02 EA

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
Effect of exercise on atherogenesis in miniature swine fed a high-cholesterol diet

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

PI:	J.S. Reitman	Clinical Associate	EA NHLBI
Other:	D.L. Fry	Chief, Laboratory of Experimental Atherosclerosis	EA NHLBI
	R.W. Mahley	Head, Comparative Atherosclerosis and Arterial Metabolism Section	EA NHLBI
	G.W. Melchior	Investigator	EA NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION
Comparative Atherosclerosis and Arterial Metabolism Section

INSTITUTE AND LOCATION
NIH/NHLBI-DIR, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

This project will determine the effect of a 10-11 month high-cholesterol, high-fat diet on the development of atherosclerosis in Yucatan miniature swine.

520

PHS-6040
(Rev. 10-76)

PROJECT DESCRIPTION

Objective: To document and describe the type and degree of atherosclerosis occurring in swine with 10-11 months of atherogenic diet.

Methods: A synthetic diet containing 15% beef tallow and 1.5% cholesterol is being fed to 9 pigs which are individually caged.

Major Findings: So far, it appears that the swine do develop atherosclerosis on this regimen which is somewhat comparable to human atherosclerosis.

Significance: The lipoprotein profile of these swine has been characterized recently by us and found to differ in minor respects from that of Hormel swine, both in the basal and cholesterol-fed state. If differences in the type and extent of atherosclerosis are also found, this would make the Yucatin swine an additional, well-characterized model available for studies on interventions such as exercise in the development of accelerated atherosclerosis.

PUBLICATIONS

1. Reitman, J.S., R.W. Mahley. Yucatan miniature swine lipoproteins: changes induced by cholesterol feeding. Biochim Biophys Acta. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02821 02 EA

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
Effect of Diazepam (Valium) on the development of experimental atherosclerosis

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

PI:	D.J. Patel	Medical Officer	EA NHLBI
Other:	J.F. Cornhill	Assistant Professor, Ohio State University	
	R.M. Nerem	Professor, Ohio State University	
	H.Y.C. Wong	Professor, Howard University	

COOPERATING UNITS (if any)
Departments of Surgery and Astronautical Engineering, Ohio State University, Columbus, OH; Department of Physiology, Howard University, Washington, DC

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION
Vascular Physiology Section

INSTITUTE AND LOCATION
NIH/NHLBI-DIR, Bethesda, MD

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)

In a pilot study the effect of Diazepam (Valium), a tranquilizer, was studied in relation to the development of experimental atherosclerosis in hyline roosters and in rabbits. The plasma cholesterol levels and the atherosclerotic lesions in the aorta were reduced in the group receiving atherogenic diet and Valium as compared to the group receiving atherogenic diet alone.

PHS-6040
(Rev. 10-76)

PROJECT DESCRIPTION

Objective: To study the effect of Diazepam (Valium), a tranquilizer on the development of experimental atherosclerosis.

Methods: A pilot study to determine if Valium could alter atherogenesis in roosters and rabbits has been completed. 1) Rooster studies: 27 birds were fed an atherogenic diet (AD) containing 2% cholesterol; 21 of these also received Valium daily, 0.2 mg/kg (11 birds) and 0.4 mg/kg (10 birds). The birds were sacrificed at the end of 8 months. Prior to sacrifice, blood pressure (BP) was obtained and blood samples drawn to determine plasma cholesterol (C) and triglyceride (TG) values. The aorta was examined for atherosclerotic lesions which were graded semiquantitatively based on 0 to 4. 2) Rabbit studies: 10 rabbits were fed AD for 5 weeks; 5 of these received daily injections of Valium (2.5 mg/kg) and 5 received daily saline injections to serve as controls. Cholesterol and TG were determined every week. The animals were sacrificed at the end of 5 weeks and the aortas examined for sudanophilic lesions.

Major Findings: In both roosters and rabbits the plasma cholesterol values and the sudanophilic lesions on the aortas were markedly reduced in the group receiving AD + Valium, compared to the control group which received AD only. In the roosters the BP prior to sacrificing was higher in the group receiving AD + Valium than the AD group; this finding remains unexplained.

Significance: There is some evidence that emotional factors may play a role in myocardial infarction. Therefore it is of interest to examine further the mechanisms associated with the decreased atherosclerosis in Valium-fed animals. To do this, further studies are needed in which the blood cholesterol values and BP are matched between the test and the control groups of animals of the same sex, age, and genetic background.

Proposed Course: The rabbit study is completed and was presented at the American Heart Association meetings (1978). Further studies are planned in roosters in which genetically matched birds of the same age and sex will be used. An attempt will be made to have matched pairs of birds with regard to plasma C and BP for the two groups -- one receiving AD + Valium and the other receiving AD only.

PUBLICATIONS

None

ANNUAL REPORT OF THE
HYPERTENSION-ENDOCRINE BRANCH
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1978 through September 30, 1979
Section on Experimental Therapeutics

The work of the Hypertension-Endocrine Branch has included numerous studies of the major vasoactive systems: i.e., 1) renin-angiotensin-aldosterone 2) catecholamines, 3) kallikrein-kinin system and 4) prostaglandins. These were done in an effort to delineate the pathogenesis of hypertension and to develop better forms of therapy for this disease.

Thirteen patients with primary aldosteronism were studied to improve our understanding of the pathophysiology of this particular form of hypertension and the means for distinguishing between bilateral hyperplasia and unilateral adenoma. Ten of the patients had adenoma while three had bilateral hyperplasia. The most definitive diagnostic test was selective venous catheterization with determination of the aldosterone/cortisol ratio from each adrenal vein and the inferior vena cava before and 15 minutes after injection of ACTH. When an adenoma was present the ratio on that side was always many times higher than that from either the inferior vena cava or the contralateral side. In patients with hyperplasia the ratios from both sides were generally equal and greater than that from the inferior vena cava. Computerized tomography was useful but it lacked precision for adenoma less than 1 cm. in diameter. The response of plasma aldosterone to upright posture has been proposed by others as a useful diagnostic test. However, while the test was correct in suggesting hyperplasia in our three patients with that disease, it indicated adenoma in only three of ten patients with that disease. Once definitive localization of the tumor is made, operative removal is simplified and operative morbidity and mortality are reduced.

Seven patients with Bartter's syndrome were studied to determine the cause of their resistance to infusions of the vasopressors angiotensin and epinephrine. Basal and post-handgrip levels of both norepinephrine and epinephrine in plasma were equal to those in a group of age-matched normal subjects. However, urinary excretion of both norepinephrine and epinephrine were increased 78% and 49% respectively above normal in the patients. This suggests increased sympathetic nervous system activity in patients with Bartter's syndrome and could explain the pressor insensitivity in these patients. Further measurements of catecholamine metabolites are underway to assure that the observed differences are not due merely to changes in metabolism.

Angiotensin converting enzyme (ACE) converts inactive angiotensin I into the potent vasoconstrictor angiotensin II and converts the potent vasodilator bradykinin into inactive fragments. Thus its overall effect is to raise blood pressure. We have made use of both a chemical assay as well as a radiochemical assay to measure ACE activity in blood, bronchial washings and urine. There were no differences in ACE activity in the blood of normals or patients

with either essential hypertension or Bartter's syndrome. This is of interest since patients with Bartter's syndrome have markedly increased levels of renin and angiotensin in their blood. Plasma ACE activity in normals was unaffected by changes in dietary sodium which produced marked changes in plasma renin activity. However, in patients with pulmonary sarcoidosis ACE activity in plasma correlated well with the activity of the disease as assessed by uptake of gallium on lung scan. The reason for this remains unclear. We have been able to demonstrate ACE activity in bronchial washings. However, there is no difference between normals and patients with pulmonary sarcoidosis and no correlation with activity of the disease. We have also been able to demonstrate ACE activity in human urine. The enzyme is inhibited by EDTA and the new specific ACE inhibitors. We await results of experiments already performed which seek to correlate urinary ACE activity with various aspects of renal function.

Urinary excretion of methoxyhydroxyphenylglycol (MHPG), a major metabolite of norepinephrine, has been proposed by others as an index of central sympathetic nervous system activity. We confirmed our earlier finding that urinary MHPG was not increased in patients with essential hypertension. We then administered separately three antihypertensive drugs with different mechanisms of action. Each of the drugs lowered blood pressure. Clonidine, which reduces sympathetic nerve activity by a central action, caused a reduction in MHPG excretion. Guanethidine, which has a peripheral sympatholytic action, also caused a reduction in MHPG excretion. Prazosin, which blocks α-adrenergic receptors pre- and post-synapse both centrally and in the periphery, caused an increase in MHPG excretion. Thus, MHPG excretion appears to offer a useful index of total and not just central sympathetic nervous system activity. Urinary MHPG did not change during the first five days of hospitalization of patients with essential hypertension at a time when they had a spontaneous and significant decrease in their blood pressure.

Highly sensitive and selective radioimmunoassays (RIA) for renal kallikrein were developed for the different enzymes in rat and man. Normal volunteers were fed diets with either 9, 109, or 259 mEq/day of salt or else they were given fludrocortisone 0.6 mg/daily for one week. The kallikrein in 24-hour collections of urine was then assayed by bioassay, and by radioimmunoassay, and by the radiochemical (TAME) esterolytic assay before and after activation with trypsin. Trypsin converts inactive prokallikrein to active or free kallikrein. The bioassay and the radiochemical assay for free kallikrein gave equivalent results. Overall the radioimmunoassay and the radiochemical assay gave similar results: i.e., urinary kallikrein was increased by a low salt diet or by administration of fludrocortisone. Both prokallikrein and free kallikrein increased with stimulation but the increases in free kallikrein were proportionately greater. The RIA appears to measure total kallikrein, i.e., both prokallikrein and free kallikrein. There was one black subject in whom free kallikrein excretion was exceptionally low on all three diets and it increased markedly with fludrocortisone. However, his excretion of prokallikrein was normal by both RIA and the trypsin -activation method.

This suggests that low levels of free urinary kallikrein may be due to failure to convert prokallikrein to free kallikrein. We have observed previously that urinary kallikrein is subnormal in blacks and in patients with essential hypertension. Studies are already underway to follow up this exciting new finding.

When we applied our new RIA for rat glandular kallikrein to rat plasma we found significant quantities of glandular kallikrein. This is contrary to accepted dogma. The antigen in plasma gave parallel cross-reactivity to the standard curve made up with rat glandular kallikrein. Glandular kallikrein is present in kidney, urine, pancreas and salivary glands. In rats that were bilaterally nephrectomized and then bled two days later the level of antigen in their plasma had increased unexpectedly 3.5 fold. When pooled plasmas were subjected to column chromatography on G-200 three peaks of immunoreactive antigen were found in plasma from either sham or nephrectomized rats. Only peak 3 reacted with the antibody in a parallel fashion with the standard and only peak 3 was increased (9 fold) in nephrectomized animals. SDS-gel chromatography shows peak 3 to have a molecular weight of 30,000 daltons $\pm$ 10%. The molecular weight for purified rat renal kallikrein (which co-chromatographs with peak 3 on G-200) is 33,000. The other peaks are probably plasma kallikrein which is immunologically different. This is the first demonstration of glandular kallikrein in blood and is an important finding now that data are forthcoming that suggest that kallikrein may be involved in the conversion of prorenin to renin in man.

We have extensively re-examined our radioimmunoassay for bradykinin in human plasma. While we have made considerable improvements in the assay and can show that the basal level of plasma bradykinin in man is probably in the picogram rather than in the nanogram range, we do not currently have the sensitivity and specificity for a reliable and reproducible assay. Therefore we are now preparing new antibody that will be N-terminal rather than c-terminal specific and, we hope, of higher titer.

A new modified tripeptide protease inhibitor was tested <u>in vitro-</u> to determine its activity as an inhibitor of kallikrein. Unfortunately, it proved to be much less active as a kallikrein inhibitor than other commonly available trypsin inhibitors. In other experiments bradykinin was shown to have no effect on platelet aggregation either alone or when o-phenanthroline, a kininase inhibitor, was added.

In collaboration with the Laboratory of Technical Development we have been able to separate mixtures of common prostaglandins in microgram quantities by means of the horizontal flow-through coil planet centrifuge. This technique has all the high resolution capability of counter-current chromatography but it makes use of simple and inexpensive equipment.

Techniques for isolation of cerebral microvessels from rats were set up in our laboratory. The techniques were modified so that a preparation of only cerebral muscular arteries could be obtained. In preliminary studies we have demonstrated that essentially only muscular arteries are obtained and that their protein synthetic activity can be easily studied.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-01822-01 HE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Catecholamine metabolism in Bartter's syndrome

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)

None

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Steroid and Mineral Metabolism

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, 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)

Patients with Bartter's syndrome are resistant to the pressor effects of angiotensin II and of norepinephrine. The observations that resistance to these vasopressors is present in patients with other disorders characterized by hypokalemia and an overproduction of prostaglandins and that pressor responsiveness to these vasopressors may be restored to normal by treatment with a prostaglandin synthetase inhibitor suggest that vasodilator prostaglandins synthesized by vascular tissue may, in part, be responsible for the pressor resistance in Bartter's syndrome. The finding of an increased excretion of norepinephrine and epinephrine in the urine of patients with Bartter's syndrome suggests that the pressor resistance to norepinephrine may be associated with an increase in activity of the sympathetic nervous system.

PHS-6040
(Rev. 10-76)

Project Description and Objective:

In previous studies, resistance to the pressor effects of angiotensin II and norepinephrine has been documented in patients with Bartter's syndrome and other disorders characterized by hypokalemia and an overproduction of prostaglandins. In patients with Bartter's syndrome, treatment with a prostaglandin synthetase inhibitor corrected the pressor resistance to angiotensin II and to norepinephrine. Vascular resistance to norepinephrine without hypotension suggests that a compensatory increase in sympathetic nervous system activity may be present. If an increase in sympathetic activity is present, as assessed indirectly by excretion of norepinephrine, epinephrine and their metabolites, then it should be restored to normal by prostaglandin synthetase inhibitors if it is the consequence of an increase in vascular synthesis of vasodilator prostaglandins.

Methods Employed:

Seven patients with Bartter's syndrome were given a diet which contained 109 mEq per day of sodium. Urine was collected daily for the determination of norepinephrine, epinephrine, normetanephrine, metanephrine and vanillylmandelic acid. After four days of control observations, indomethacin 150 mg/day was given for four days. The control values are compared to values for seven normal age-matched subjects.

Major Findings:

Preliminary results for urinary norepinephrine and epinephrine in seven age-matched normal subjects and in seven patients with Bartter's syndrome are presented below as means $\pm$ SE.

	Normal subjects µg/day	Bartter's syndrome µg/day
Norepinephrine	73.3$\pm$11.2	130.4$\pm$23*
Epinephrine	27.8$\pm$3.4	41.5$\pm$3.0*

*$P<0.01$

The results indicate that patients with Bartter's syndrome have significantly higher values for urinary norepinephrine and epinephrine, a finding consistent with an increase in sympathetic nervous system activity. To be certain that the increase in urinary norepinephrine and epinephrine is not the result of an alteration in their metabolism, major urinary metabolites will be measured. The effects of treatment with a prostaglandin synthetase inhibitor on urinary norepinephrine, epinephrine and their major metabolites will also be determined.

Publications:

1. Radfar, N., Gill, Jr., J.R., Bartter, F.C., Bravo, E., Taylor, A.A., and Bowden, R.E.: Hypokalemia, in Bartter's syndrome and other disorders, produces resistance to vasopressors via prostaglandin overproduction. Proc. Soc. Exp. Biol. Med. 158: 502-507. 1978.

2. Gill, Jr., J.R. and Bartter, F.C.: Evidence for a prostaglandin-independent defect in chloride reabsorption in the loop of Henle as a proximal cause of Bartter's syndrome. Amer. J. Med. 65: 766-772, 1978.

3. Gullner, H.G., Gill, Jr., J.R., Bartter, F.C., Chan, J.C.M., and Dickman, P.S.: A familial disorder with hypokalemic alkalosis, hyperreninemia, aldosteronism, high urinary prostaglandins and normal blood pressure that is not Bartter's syndrome. Trans. Ass. Am. Phys., in press.

4. Gill, Jr., J.R.: Neural control of renal tubular sodium reabsorption. Nephron 23: 116-118, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-01823-01 HE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (30 characters or less)

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
OTHER:	John L. Doppman, M.D.	Chief, Diagnostic Radiology Department	DR CC

COOPERATING UNITS (if any)

J.A. Oates, M.D., Department of Pharmacology, Vanderbilt School of Medicine, Nashville, Tennessee

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Steroid and Mineral Metabolism

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	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)

Studies in patients with primary aldosteronism have been directed toward improvment of techniques for diagnosis and understanding the pathophysiology of the disorder. As primary aldosteronism may be produced by either bilateral adrenal hyperplasia or by unilateral adenoma, a distinction between the two adrenal abnormalities is necessary because patients with hyperplasia respond poorly to operation. The response of plasma adrenal corticosteroids to postural change, computed tomography of the adrenal glands and selective adrenal vein sampling for determination of adrenal corticosteroids are the diagnostic modalities currently under evaluation for their usefulness in identification of patients with primary aldosteronism caused by an aldosterone-producing adenoma. The results to date indicate that selective adrenal vein sampling is the most reliable technique for evaluation of adrenal function in primary aldosteronism. The effects of an overproduction of aldosterone on the production of prostaglandins by the kidney and the effects of treatment with a prostaglandin synthetase inhibitor on renal function and on adrenal steroid biogenesis in patients with primary aldosteronism are also under study.

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Project Description and Objective:

Published studies of patients with primary aldosteronism indicate that if, after three hours of walking or standing, plasma aldosterone is lower than the value obtained three hours earlier at bedrest, an adrenal adenoma is the probable cause of the aldosteronism. The reported experience with this procedure has indicated that the number of false positives is very low and this has resulted in an extensive use of this test to select patients for operation. To determine the extent to which this test gives false negative results, it was evaluated in terms of the results of computed tomography of the adrenal glands and selective adrenal vein catheterization.

In patients with Bartter's syndrome, aldosteronism and hypokalemia are associated with hyperreninemia, overproduction of prostaglandins by the kidney and normal blood pressure and treatment of these patients with a prostaglandin synthetase inhibitor produces sodium retention. In patients with primary aldosteronism, aldosteronism and hypokalemia are associated with suppressed plasma renin activity and hypertension. If these differences in plasma renin activity and blood pressure in the patients with primary aldosteronism are associated with differences in the renal excretion of prostaglandins or in differences in the renal response to a prostaglandin synthetase inhibitor, the findings may be helpful in understanding the role of prostaglandins in renal function.

Methods Employed:

Thirteen patients with primary aldosteronism were given a diet which contained 109 mEq per day of sodium, urine was collected daily for the determination of aldosterone, 17-hydroxycorticosteroids, 17-ketosteroids, and prostaglandin E_2. Control observations included measurement of plasma renin activity, plasma aldosterone and cortisol at bedrest and after three hours of walking or standing. Computed tomography of the adrenal glands and selective adrenal vein catheterization for measurement of aldosterone and cortisol before and fifteen minutes after 25 units of ACTH (1-24 peptide) were also performed. Then the patients were treated with indomethacin 150 mg/day for six to eight days and the control observations were repeated.

Major Findings:

The results of selective adrenal vein catheterization are presented as a ratio of aldosterone/cortisol in the table below; the values are means $\pm$ SE for each.

	Basal Aldo/Cortisol Ratio			Post-ACTH Aldo/Cortisol Ratio		
Adenoma (N=10)	Right Adrenal Vein	Left Adrenal Vein	Vena Cava	Right Adrenal Vein	Left Adrenal Vein	Vena Cava
Left (N=7)	1.7±0.5	124±88	3.7±1.8	0.9±0.3	481±465	3.6±0.8
Right (N=3)	20±16	1.5±0.5	1.4±0.2	16±10	0.8±0.6	1.8
Hyperplasia(N=3)	8.3±4.5	8.3±1.9	0.8±0.03	11.1±2.7	9.1±5.1	2.0±0.5

In the ten patients in whom a high aldosterone/cortisol ratio for one adrenal was associated with a contralateral ratio equal to or lower than the ratio for the vena cava, an adenoma was found at operation. In only three of these ten patients did the response of plasma aldosterone to standing correctly predict the presence of adenoma; in none of the three patients with hyperplasia was an adenoma predicted. Thus, although the response of plasma aldosterone to standing in the patients with primary aldosteronism did not wrongly predict the presence of an adenoma, it failed to indicate the presence of an adenoma in 70 percent of the patients and is, therefore, a poor criterion for the selection of patients for operation. Selective adrenal vein catheterization, however, has proved to be a very effective means for distinguishing adrenal adenoma from hyperplasia and represents a major advance in evaluation of the patient with primary aldosteronism. Also, preoperative localization of the tumor has simplified its operative removal, with a reduction in operative mortality and morbidity. Computed tomography of the adrenal glands has also been helpful in detecting adrenal adenomas, but it lacks the precision of selective adrenal vein catheterization, as it is presently unable to demonstrate an adenoma less than 1 cm in diameter, and depends on the presence of retroperitoneal fat to delineate the adrenal clearly.

Publications:

1. Dunnick, N.R., Schaner, E.G., Doppman, J.L., Strott, C.A., Gill, J.R., and Javadpour, N.: Computed tomography in adrenal tumors. Amer. J. Roentgenology 132: 43-46, 1979.

2. Dunnick, N.R., Doppman, J.L., Mills, S.R., and Gill, Jr., J.R.: Preoperative diagnosis and localization of aldosteronomas by measurement of corticosteroids in adrenal venous blood. Radiology, in press.

3. Auda, S.P., Brennan, M.F., and Gill, Jr., J.R.: Evolution of the surgical management of primary aldosteronism. Annals of Surgery, in press.

4. Dusing, R., Gill, J.R., and Bartter, F.C.: Prolactin in primary aldosteronism. Clin. Sci. 56: 381-383, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01909-05 HE

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

INFORMATION RETRIEVAL IN PHARMACOLOGY

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

P.I.:	Elise A. B. Brown, Ph.D.	Research Pharmacologist	HE NHLBI
OTHER:	Harry R. Keiser, M.D.	Deputy Chief, Hypertension-Endocrine Branch	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, 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)

Information retrieval and appraisal activities have been established for the broad areas of biochemical pharmacology, therapeutics and toxicology. This project aids new research by determining the amount of knowledge already available on biomedical problems and by examining the procedures available for solving problems. Sources of information include computer-assisted library searches, consultations with knowledgeable individuals and analyses of a problem as to its more efficient solution.

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Objectives: This project facilitates the initiation and continuation of both laboratory and clinical research projects by determination of the current knowledge about pharmacological topics and by location and assessment of the methods most suitable for solving the problem. Both patient-care physicians and laboratory scientists need knowledge of drug interactions and toxicological data for their experimental projects.

Methods: Both chemical and clinical information need to be readily available in order to satisfy the complex nature of modern biomedical research. Research procedures by their nature are not standardized but are changed to suit the nature of the problem. A large portion of the background material for biomedical information is contained in the Medline, Toxline, and other files of the National Library of Medicine. Physical and chemical data and additional biomedical information are obtained on line from the computerized files of the Lockheed Dialog Service through the Chemical Abstracts, Biological Abstracts, Scisearch or Inspec-physics files from 1970 to date. We consult with knowledgeable individuals, make direct and computer-assisted library searches and provide scientific expertise as requested to other individuals in the intramural program of NHLBI.

Results: More than 60 investigators used the services of this project. The reference librarians refer several investigators each year who seem to have difficult problems in our area of expertise which the librarians cannot resolve. A notice was sent to all investigators in the intramural program of this institute in order to inform them of the scope of our services. Most users consult us several times and refer others to us.

Our searches are not routine; our purpose is to find information which is difficult to access or to supply information rapidly. We have trained six investigators who desired to do their own computer searches in their speciality. At least two review articles were written with our aid this year. Our intent is to determine exactly what information an investigator needs and to obtain pertinent information without a lot of extraneous material. Our objective is to answer questions precisely, not to get large bibliographies.

We have taken advanced training both in medline data bases and in the use of Chemical Abstracts data bases.

Major areas of interest this year include:

1. Prostaglandins
2. Toxicolgic problems; nitrosomines, TCDD, etc.
3. Hypertension, treatment, drugs
4. Angiotensin, bradykinin, kallikrein
5. Atherosclerosis, etiology
6. Receptor mechanisms, endorphins
7. Allergy, SRS-A

Significance to Biochemical Research: In the last thirty-five years, a large mass of biomedical and physical-chemical information has been generated. More efficient methods were needed to assess the current status of a problem. This project speeds up access to the medical and chemical literature. It saves time of expensive personnel by making information available rapidly. It helps to assure that projects are not repetitious and it assesses methods so the most efficient one can be chosen.

Proposed Course: We propose getting both another cathode-ray-tube terminal and a new hard copy terminal. The current equipment is utilized over 70% of the time so that delays are encountered frequently in getting access to a terminal. The facilities are shared with purchasing personnel whose needs will increase. The demand for use of automated information data bases, for storage of data in computer files, for use of computer programs for computation and for manuscripts increases yearly in clinical and toxicological research.

Publications:

1. Brown, E. A. B. and Maling, H. M. The effects of paraquat and related herbicides on the acetylcholinesterase of rat lung. Biochemical Pharmacology, 1979 in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01918-02 HE

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

Urinary Methoxyhydroxyphenylglycol excretion as an index of sympathetic nervous system function.

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

PI:	David Horwitz, M.D.	Senior Investigator	HE NHLBI
OTHER:	Joseph L. Izzo, M.D.	Staff Associate	HE NHLBI
	Harry R. Keiser, M.D.	Deputy Chief, Hypertension-Endocrine Branch	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:
0.50	0.50	

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)

Urinary excretion of MHPG reflected the magnitude of blood pressure reduction caused by the antihypertensive agents clonidine and guanithidine but did not correlate with spontaneous changes in average blood pressure measurements during the initial week of hospitalization of patients with essential hypertension.

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PHS-6040
(Rev. 10-76)

Objectives: Methoxyhydroxyphenylglycol (MHPG) is a major metabolite of the sympathetic neurotransmitter norepinephrine. The present study was concerned with evaluating the usefulness of the urinary excretion of MHPG as an index of integrated activity of the sympathetic nervous system over the course of a day. Two types of observations were made: 1) changes in excretion of MHPG during spontaneous or drug-induced changes in blood pressure, and 2) a comparison of responses of MHPG with other biochemical indices of sympathetic nervous system activity.

Methods: Hospitalized patients with mild to moderate essential hypertension received a diet containing 109 meq/day of salt, had their blood pressure monitored four times daily by the arm-cuff method and collected daily 24 hour urine samples. Urinary MHPG was measured by a modification of the method of Dekirmenjian and Maas. Conjugates were hydrolyzed by β-glucuronidase/aryl sulfatase and MHPG extracted with ethyl acetate. MHPG- trifluoroesters formed by reactions with trifluoroacetic anhydride were then assayed by gas chromatography. VMA was determined by the method of Pisano.

Results:

A. Excretion of MHPG During Spontaneous Blood Pressure Reduction. A group of nine hypertensives who showed declining blood pressure during their first five hospital days was studied. The average of four daily blood pressure measurements for all subjects and the average of 24 hour values for excretion of MHPG on the first hospital day were compared with corresponding values for the fifth hospital day. Whereas average blood pressure fell 13/8 (Systolic/Diastolic) Torr there was no reduction in average excretion of MHPG.

Day	MHPG excretion mg/day	Blood pressure Torr Systolic	Diastolic
1	1.9	145	103
2	2.0	132	95

In order to evaluate the extent to which blood pressure falls during the first week of hospitalization reflected decreased levels of physical activity and removal from occupational stresses, two groups of consecutively admitted patients were managed equivalently, except that one group remained on the ward throughout the day whereas the other patients left the ward to pursue their usual job schedules. There was no material difference in the average blood pressure levels of the two groups.

AVERAGE DAILY BLOOD PRESSURE mm Hg.

GROUP	DAY 1	2	3	4	5
SYSTOLIC					
Working n=9	143	138	134	130	131
Not Working n=13	144	136	135	132	133
DIASTOLIC					
Working n-9	103	102	98	96	96
Not Working n=13	101	98	97	94	92

Data for MHPG are too incomplete for analysis at this time.

B. Effects of Antihypertensive Medications on Excretion of MHPG. Effects of three antihypertensive agents with different mechanisms of action were studied; clonidine (considered to reduce sympathetic nerve traffic by a central effect) guanethidine (induces peripheral sympatholysis), and prazosin (blocks pre- and post-synaptic blocks α-adrenergic receptors. The changes in excretion of MHPG were consistent in direction with those anticipated from the mechanisms of action of the drugs. Changes in excretion of VMA were similar to those of MHPG. Results are shown in the appended table.

Significance of findings: MHPG appears to offer a useful index of sympathetic nervous system activity integrated over a 24 hour period. It is more stable and more readily measured than norepinephrine and correlates with other indices. The lack of correlation of blood pressure fall and MHPG excretion in the first days of hospitalization is under study and may result from limited sensitivity of the test or may reflect the process of patients becoming progressively more accustomed to having their blood pressure measured; i.e. initial blood pressure measurements after hospitalization may be unrepresentative of the true integrated blood pressure curve.

Proposed Course of Study: The correlation of MHPG excretion with other indices and with spontaneous blood pressure measurements will be pursued.

Publications:

1. Izzo, J. L.; Horwitz, D. and Keiser, H.R. Reduction in human urinary MHPG excretion by guanethidine: Urinary MHPG as an index of sympathetic nervous activity. Life Sciences 24: 1403, 1979.

2. Patel, D. and Horwitz, D. Role of psychophysiologic techniques in the treatment of hypertension. Urban Health 8: 46, 1979.

EFFECTS OF ANTIHYPERTENSIVE AGENTS ON BLOOD PRESSURE MHPG, VMA, AND PLASMA NOREPINEPHRINE

Drugs	n	Blood Pressure (Standing) Systolic		Diastolic		MHPG Mg/day		VMA Mg/day		Plasma Norepinephrine (ng/ml) Lying		Standing	
		Control	Drug	Control	Drug	Control	Drug	Control	Drug	Control	Drug	Control	Drug
Guanethidine	6	138	116*	98	80*	1.8	0.8*	4.3	2.9*	-	-	-	-
Glonidine	5	132	104*	98	80*	1.3	1.1*	4.4	3.0*	N.A.	N.A.	N.A.	N.A.
Prazosin	6	143	133*	101	94*	1.9	2.6	5.4	5.9	0.29	0.58	0.48	1.04*

* $p < .05$
N.A. Collected but not assayed

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01919-02 HE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Re-evaluation of Bradykinin Assay and Methods of Collection

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

P.I.:	Michael Fordis, M.D.	Staff Associate	NHLBI HE
OTHER:	Harry R. Keiser, M.D.	Deputy Chief, Hypertension-Endocrine Branch	NHLBI HE

COOPERATING UNITS (if any)

None.

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, Bethesda, maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.75	0.75	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The investigation of the plasma kinin assay was continued. Topics involved in study included alternative methods of plasma preparation, alternative methods of separation of bound from free antigen, specificity, buffer system, surface adherence, purification of tracer, modification of volume, statistics, and assay parameters. The problems with the lack of specificity and with the low antibody affinity for bradykinin could not be overcome. An acceptable plasma kinin assay will await the production of new antibody. The dog hind limb bioassay for kinin was examined. Specificity of the vasodilatation response could not be shown to be due to bradykinin alone. Neither vasoactive monoamines nor acetylcholine appeared to be responsible for the nonspecific responses. Finally chromogenic kallikrein substrate suggested to be a bradykinin receptor blocker was examined in the dog hind limb. No effect on vasodilatation was observed. In another experiment a protein inhibitor was examined as a possible kallikrein antagonist. While the inhibitor is small, nonimmunogenic, and can enter cells, its potency as a kallikrein antagonist is too low to be of practical use currently.

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I. Bradykinin Plasma Assay

A. Objective: In the current study we continue our evaluation of assay methods for bradykinin in blood.

B. Methods and Results:

1. Buffer System - Experiments were designed to determine the pH optimum for antigen-antibody binding. Additional experiments were performed to select a buffering system whose capacity was greatest in the range of maximal binding. A citric acid-sodium citrate buffering system at pH 6.2 yielded maximal binding.

2. Surface Adherence - Despite the use of siliconized glassware and plastic tubes, adherence of bradykinin to surfaces is of concern. Experiments with various concentrations of albumin and lysozyme were performed to determine their effect on surface adherence and on antigen-antibody binding. A concentration of 0.1% albumin produced the greatest inhibition of surface binding with minimal inhibition of antigen-antibody binding.

3. Modification of Volumes - To decrease the dilution of sample reagent volumes were reduced in half. Antibody titration curves were performed and antibody dilutions adjusted for the new volumes: 150 µl of antibody, 100 µl of iodinated bradykinin, and 200 µl of sample, standard, or buffer.

4. Purification of Tracer - Iodinated bradykinin is prepared by the chloramine-T method of New England Nuclear. Sodium iodide is removed by Dowex chromatography. Tracer immunoreactivity was not increased either by additional Sephadex chromatography or by reductions from 15 1/2 minutes to 2 minutes in reaction time for iodination.

5. Parellelism - When the samples were diluted, the curves produced were often not parallel to the standard curve. Nonparallelism can be produced by both nonspecific as well as specific inhibitors of antigen-antibody binding. The nonspecific etiologies of heparin, pH changes, radioactive contamination, and salt effects did not explain the non-parallelism. On the other hand, kininogen I may act as a specific inhibitor of antigen-antibody binding. Removal of kininogen I is required for both reliable kinin measurements as well as achieving parallelism. Methods involving ethanol precipitation of blood followed by organic extractions may work better for removal of kininogen I than methods with TCA precipitation of blood and IRC-50 chromatography. Difficulties with the latter methods have been discussed in a previous report. Hulthen's method, typical of the former, involves ethanol precipitation, concentration, acidification, and extraction against methylene chloride. Since there is no readily available assay for kininogen I, the low molecular weight kininogen, the high molecular weight kininogen (HMWK) was measured in an assay developed by Dr. David Proud. The HMWK provided an index of kininogen contamination. Our samples had up to 5 ng of HMWK present. What this degree of contamination represents is

unclear. Kininogen contamination is present. How much kininogen I is present is unknown as is the extent with which it will cross-react with the kinin antibody.

6. Separation of Bound Antigen - Millipore filtration has inherent variability in differences in the filters from lot to lot and from one filter to the next. As a result an alternative method of separation was considered, that of double antibody separation. Double antibody titration curves with rabbit anti-sheep IgG were performed. The effects of pH, temperature, incubation time, and mixing were studied. Conditions were chosen to maximize antigen-antibody binding and separation of bound from free. Antigen-antibody bindings were on the order of 20% with the usual antisera for bradykinin and 30% with antisera purified for the anti-bradykinin antibody. Assays where this separation technique was used often has unacceptable high levels of coefficient of variation. The double antibody technique is not a practical alternative to our previous method for separation.

7. Plasma Separation - Various plasma purification methods have been proposed. Precipitation with trichloracetic acid followed by IRC-50 chromatography is widely used. The specificity problems with this method have been outlined in the previous report. Many of the other methods involve ethanol precipitation followed by organic extractions. Hulthen's method described above is typical of many and represents a modification of Mashford's method. This procedure yields an overall recovery of radioactive tracer of 45.1 $\pm$ 7.1% (mean $\pm$ S.D.). Bradykinin values are generally less than one nanogram and are often undetectable. Recovery of cold bradykinin is 62.8 $\pm$ 25.8% (mean $\pm$ SEM). This scatter is unacceptable. Furthermore as mentioned above there is some kininogen contamination of these extracts.

8. Statistics - The Rodbard Program was used for plotting the data. The program uses a weighting procedure to deal with variances which change with dose. Experiments were done to evaluate the effect of dose changes on variance. The variance data were analyzed and the appropriate parameters calculated for use in Rodbard's program. The method of Grubbs (1969) for detection of outliers was used. A conservative α of 0.01 was used. Despite these changes the assay parameters discussed below were changed little.

9. Assay Parameters - In the previous report problems with sample preparation and radioactive contamination with Vinci's method were addressed. Based on his minimal detectable concentration (MDC); preparatory procedures for 2 ml of plasma; sample size and his recoveries, the MDC in plasma was 1.2 - 3.1 ng/ml plasma. Our reported values suggest that this MDC may be outside the physiological range. Despite continued experimentation our current limits for MDS are in the 31 to 62 pg/tube range and occasionally drop into the 7-15 pg/tube range. With current methods of preparation and recoveries this translates to 39 - 78 pg/ml blood. Interassay variability is 17.8% and intra-assay variability is 7 - 10%. Near the limits of detection the coefficient of variation is about 20% based on 4 replicates.

II. Bioassay

The dog hindlimb was used to bioassay bradykinin.

A. Methods: Blood samples were prepared according to Binia. Blood was precipitated in ethanol and centrifuged. The supernatant was concentrated, acidified, extracted against butanol, and lypholized overnight.

For purposes of assay a 20 kg foxhound was anesthetized and an electromagnetic flow probe was placed on the femoral artery. Above the flow probe, a catheter was fed retrograde into the femoral artery. The most stable baselines were obtained when the muscle surrounding the flow probe was removed. Catheter dead space was 0.1 cc or less. All samples and standards were injected in volumes of 0.5 cc and followed by a normal saline rinse of 0.5 cc. The vasoactive effect of sample or standard was recorded as the change in flow through the femoral artery.

The threshold of detection of bradykinin standard ranged from 0.5 to 2.0 ng.

B. Results:

1. Bradykinin Potentiation - Experiments were performed to evaluate the potentiation of converting enzyme inhibition on the vasodilatation produced by bradykinin. The proline derivative, SQ14,225 was given by bolus injection both intra-arterially and intravenously in doses of 10 mg/kg. No effect on vasodilatation by bradykinin was observed. On the other hand, marked potentiation of bradykinin effect was observed when the nonapeptide SQ20,881 was infused. SQ20,881 was administered in doses of 300 µg/kg bolus i.v. followed by an injection of 10 µg/kg/min. The threshold dose for bradykinin effect dropped from 2 ng to 0.5 ng in one animal and from 0.5 to 0.125 ng in another.

2. Plasma Samples - Injection of samples of extracted plasma produced measurable vasodilation. Incubation of samples with carboxy peptidase B (12 to 125 µ) at 37°C for 15 minutes to 60 minutes did not destroy the vasodilatory effect. The effect of bradykinin standard was destroyed by incubation with carboxypeptidase B under the same conditions. The vasodilatory effect of extracted plasma was destroyed by incubation with carboxypeptidase ß. The vasodilatory effect of extracted plasma was not blocked by atropine in doses up to 3.6 mg intra-arterially. Doses of 2.4 mg atropine i.a. blocked up to 500 ng of acetylcholine. Similarly diphenhydramine in doses up to 50 mg did not abolish the vasodilatory effect of extracted plasma. Diphenhydramine in a dose of 20 mg would abolish the vasodilatory effect of 500 ng of histamine. The vasodilatory substances appear not to be bradykinin, acetylcholine or histamine. Serotinin while possible is less likely because platelet poor plasma was used. Also the extraction which removed histamine should remove serotonin because of the similarity of their pH's. The nature of the vasodilatory substance awaits further investigation.

3. Chromogenic Kallikrein Substrate - The chromogenic kallikrein substrate S2303 may block the bradykinin receptor on guinea pig ileum. In concentrations up to 10^{-2} M given as a 1.0 ml bolus into the femoral artery, no effect on the vasodilatory effect of an injection of 6 ng of bradykinin standard was observed.

C. Plan: The physiological levels of bradykinin are probably in the picogram range. Measurement of bradykinin in plasma is complicated by cross-reaction of antibody with the carboxy-terminal bradykinin of kininogen I. This may well explain the high plasma levels measured by many methods. Since most kinin antibodies have carboxy-terminal specificity secondary to the techniques used for antibody production, most of the cross-reacting kininogen I must be removed. With current techniques this is difficult. Furthermore many radioimmunoassays are not sensitive enough to measure kinin in the physiological range. Our next step is to make a new anti-bradykinin antibody with amino-terminal specificity.

Publications:

1. Vinci, J.M., Horwitz, D., Zusman, R.M., Pisano, J.J., Catt, K.J., and Keiser, H.R.: The effect of converting enzyme inhibition with SQ20,881 on plasma and urinary kinins, prostaglandin E, and angiotensin II in hypertensive man. Hypertension 1979 (In press).

2. Vinci, J.M., Zusman, R.M., Izzo, J.L., Jr., Bowden, R.E., Horwitz, D., Pisano, J.J., and Keiser, H.R.: Human urinary and plasma kinins: Relationship to sodium-retaining steroids and plasma renin activity. Circulation Research 44 (2): 228-237, February 1979.

3. Horwitz, D., Margolius, H.S., and Keiser, H.R.: Effects of dietary potassium and race on urinary excretion of kallikrein and aldosterone in man. J. Clin. Endocrin. Metab. 47: 296-299, 1978.

4. Vinci, J.M., Zusman, R.M., Izzo, J.L., Jr., Bowden, R.E., Horwitz, D., Pisano, J.J., and Keiser, H.R.: Relationship of human urinary and plasma kinins to sodium-retaining steroids and plasma renin activity. In: Proceedings of Tokyo International Symposium on Kinins, Tokyo, Japan, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01921-02 HE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Angiotensin Converting Enzyme Activity in Sarcoidosis, Hypertension, and Normal Volunteers.

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
Hypertension-Endocrine Branch

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Section on Experimental Therapeutics

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Angiotensin converting enzyme (ACE) converts angiotensin I (A^{I}) to angiotensin II (A^{II}) and inactivates bradykinin (BK) by its dipeptidyl-carboxypeptidase activity. It is thus possible that ACE has an important role in the regulation of blood pressure in normal physiology. The factors modulating serum ACE activity and its relationship to tissue ACE activity are unclear. No abnormalities of serum ACE activity have been reported in hypertensive subjects. Serum ACE-activity has previously been reported to be supranormal in sarcoidosis and Gaucher's disease but the increases in serum ACE-activity were not associated with elevations of blood pressure. Serum ACE-activity was measured in normal volunteers, hypertensive subjects on 9 mEq and 109 mEq sodium diets and in patients with sarcoidosis. The levels of serum ACE activity were similar in normal and hypertensive subjects and were not influenced by dietary sodium. Serum ACE-activity in patients with sarcoidosis varied with the activity of the disease.

PHS-6040
(Rev. 10-76)

Objectives: Angiotensin converting enzyme (ACE) converts angiotensin I (A^{I}) to angiotensin II (A^{II}), a potent vasopressor and steroidogenic hormone. ACE also inactivates bradykinin (BK), a potent vasodilator. The enzyme is a dipeptidyl carboxypeptidase. ACE has been isolated and characterized and it is identical to kininase II. It is present in both serum and endothelial cells. There is a positive correlation between tissue vascularity and extractable ACE. Both the factors modulating ACE's release into serum and the relationship between tissue ACE-activity and serum ACE-activity are poorly understood. It has been reported that the highest serum concentrations of ACE are present in patients with either active sarcoidosis or Gaucher's disease.

The present study was undertaken to: 1) establish an assay for ACE in our laboratory, 2) study ACE activity in blood of normals and hypertensives to see if the enzyme might be involved in the pathogenesis of hypertension, and 3) study ACE activity in urine, bronchial washings and tissue of certain patients to see how ACE activity in these sources correlate with levels in blood.

Methods: All samples were assayed via the Ventrex Method, a radiochemical assay previously described in Project Number ZO1 HL 01921-01 HE (1978).

Biochemical characterization studies of the angiotensin converting enzyme in urine and bronchial washings were assayed by modifications of the spectrophotometric method of Cushman and Cheung. In this method hippuryl-histidyl-leucine, an artificial substrate, is cleaved generating hippuric acid and His-leu. The hippuric acid generated is extracted with ethyl acetate, which is removed by evaporation. The hippuric acid is dissolved in 1 N NaCl and quantified spectrophotometrically at 228 nm.

There was no consistent difference in ACE activity in bronchial washings from normals and patients with sarcoidosis.

Urinary angiotensin converting enzyme in 24 hour urine collections of normal volunteers and patients with Bartter's syndrome on either 9 mEq or 109 mEq of salt per day is variable. Correlations with urinary electrolytes and serum ACE activity are being determined.

ACE activity in urine and bronchial washings is inhibited by EDTA and SQ14,225 and SQ20,881. The latter two compounds are specific inhibitors of ACE activity.

Clinical Study: The following groups of patients have been evaluated for serum ACE activity: a) hypertensives (n=30), b) normal volunteers (n=25), c) patients with systemic sarcoidosis (n=60), d) patients with Bartter's syndrome (n=7). Normal and hypertensive subjects were studied while on either 9 mEq or 109 mEq of salt per day. Samples were collected while these subjects were supine or standing. Samples of serum were collected at random from patients with sarcoidosis.

Results: There was no consistent difference in serum ACE activity between normals, hypertensives and patients with Bartter's syndrome.

Serum ACE activity was not influenced by dietary sodium.

Serum ACE activity in patients with sarcoidosis was positively correlated with disease activity as reflected by uptake of gallium on lung scans.

Serum ACE activity is not correlated with ACE activity in bronchial washings in both normal volunteers and patients with sarcoidosis.

Significance of findings: Serum ACE activity can be measured quickly and accurately in our laboratory via the Ventrex method.

The lack of difference in serum ACE activity between normals and patients with essential hypertension suggests that ACE is not involved in the pathogenesis of that disease.

Serum ACE activity can be measured in patients with sarcoidosis and it appears to correlate with disease activity.

ACE activity can be measured in bronchial washings from patients with sarcoidosis but it does not correlate with activity of the disease.

Urinary ACE activity is now measurable in our laboratory.

Serum ACE activity is normal in patients with Bartter's syndrome in spite of elevated levels of serum renin, Angiotensin II and aldosterone.

Proposed Course of Project: There are now several peptides which inhibit ACE activity and one of them is SQ14,225 which is orally active and reduces blood pressure in a variety of hypertensive subjects. We will measure serum ACE activity in subjects treated with SQ14,225 and attempt to correlate blood pressure response with changes in ACE activity and with other vasoactive substances, i.e., kallikrein-kinin and prostaglandins.

We will measure urinary ACE activity in normal volunteers on 9 mEq and 109 mEq salt diets and determine its relationship to urinary kallikrein and urine electrolytes.

ACE activity measured in urine and bronchial washings will be further characterized and compared biochemically with the serum enzyme; optimal pH chloride concentration and molecular weights will be determined.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01922-01 HE

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

SEPARATION OF PROSTAGLANDINS WITH THE HORIZONTAL FLOW-THROUGH COIL PLANET CENTRIFUGE

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

P. I.:	Elise A. B. Brown, Ph.D.	Research Pharmacologist	HE NHLBI
OTHER:	Harry R. Keiser, M.D.	Deputy Chief, Hypertension-Endocrine Branch	HE NHLBI
	Yoichiro Ito, M.D.	Medical Officer	TD NHLBI

COOPERATING UNITS (if any)

Laboratory of Technical Development, NHLBI

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Experimental Therapeutics

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, 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)

Prostaglandins, thromboxanes and their metabolites have been separated using the new horizontal flow-through coil planet centrifuge (Ito, unpublished) to perform countercurrent chromatography. This continuous flow system provides separations of compounds in a manner similar to column and thin layer chromatography without the use of solid supports. Separations are produced in Teflon tubing wound helically around a supporting rod. A two phase solvent system is used with one of the two phases stationary while the other phase flows continuously due to a low pressure metering pump. Microgram quantities of material have been separated. The elution sequence of 6-keto-$PGF_{1\alpha}$, thromboxane B_2, $PGF_{2\alpha}$, PGE_2, 13,14-dihydro-15-keto-$PGF_{2\alpha}$, PGB_2 and PGA_2 occurs when the aqueous phase of chloroform: acetic acid: water (2:2:1) is the mobile phase.

551

Objectives: The purpose of this project is to effect the separation of several varieties of prostaglandins and thromboxanes as well as other derivatives of arachidonic acid and eicosatrienoic acid. For this purpose, the new horizontal flow-through coil planet centrifuge developed by Dr. Y. Ito (Laboratory of Technical Development) has been used. This continuous flow system provides separations of compounds in the same theoretical manner as countercurrent chromatography, thin layer chromatography, and high pressure liquid chromatography without the use of solid supports. It is an automated form of countercurrent chromatography modernized to eliminate the cumbersome glass apparatus which has been used before.

Methods: The horizontal flow-through coil planet centrifuge has a pair of coiled separation columns. One column enables preparative-scale separations and the other, analytical scale separations, with high partition efficiency. The separations which are reported here were made with a PTFE coiled tube, 0.55 mm in diameter and 1/4" helical diameter with a total capacity of about 24 ml. The column was mounted on the analytical side. The sample rotation rate was 500 rpm clockwise and flow rates of both 6 ml/hour and 2.4 ml/hour were used regulated with a Chromatronix pulse free, metering pump. Samples were collected into counting vials at 10 minute intervals (1 ml or 0.4 ml). All compounds utilized were radiolabeled either with 14-C or 3-H and the carriers added were gifts of Dr. John Pike of Upjohn Company, Kalamazoo, Michigan.

Results: The solvent system of chloroform: acetic acid: water (2:2:1) was used for all separations. The majority of the separations were carried out with the upper aqueous phase mobile and the lower chloroform phase stationary. If the lower phase is mobile, the reverse elution sequence of compounds occurs. We have been able to separate the major prostaglandin and thromboxane classes in microgram amounts from each other into narrow bands of 1 to 3 ml. These compounds separate in the following order when the aqueous phase is mobile; 6-oxo-$PGF_{1\alpha}$, thromboxane B_2, $PGF_{2\alpha}$, PGD_2, PGE_2, 13,14-dihydro-15 keto-$PGF_{2\alpha}$, 13,14-dihydro-15-keto-PGE_2, PGB_2, PGA_2, PGA_1. We have had similar separations whether the total volume of the columns were 24 or 250 ml. The system is versatile and it is easy to change the scale of separations. Elution patterns are highly reproducible and the same column can be used an unlimited number of times.

Significance to Biochemical Research: The information on prostaglandin formation, degradation and action as local hormones has exploded to as many as 300 references per month. The importance of prostaglandins in kidney function, in the etiology of hypertension and thrombus formation is still being assessed. This separation technique should be useful for isolating compounds after synthetic procedures and for looking at a 'spectrum' of compounds which may occur in tissues. The advantages of this method are that the column is reuseable, can be calibrated, does not utilize a solid support, and is inexpensive relative to mass fragmentography and high-speed-liquid chromatography. The samples can be totally recovered if a separation fails to occur, concentrated and a new separation performed. At the end of a run the column contents can be eluted and any residual substances analyzed.

Proposed Course: We propose to extend our work to mixtures of known composition and to biological samples. We will also assess other solvent systems. We will extend our studies to other classes of compounds. We will explore other monitoring and detection devices such as radioactive flow counters, fluorescent detectors and electrolytic detectors.

Publications:

1. Elise, A. B. Brown and Yoichiro Ito. Separation of prostaglandins with the new horizontal flow-through coil planet centrifuge. The Pharmacologist 21(2). 1979 (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01923-01 HE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Platelet Aggregation with Kinin

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

P.I.:	Michael Fordis, M.D.	Staff Associate	NHLBI HE
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COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.05	0.05	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The effect of bradykinin on platelet aggregation was examined. Bradykinin did not initiate nor promote platelet aggregation. The addition of 1,10 phenanthroline, a kininase inhibitor, also had no effect on platelet aggregation.

554

Objectives: it has been demonstrated that certain prostaglandins, PGE_2, and $PGF_{2\alpha}$ are formed and released during clotting. Arachidonic acid can induce the release reaction of PGE and initiate aggregation. Bradykinin acts on phospholipase to increase release of arachidonic acid. Arachidonic acid comprises nearly twenty percent of platelet fatty acids. It may be that kinin formed when kallkrein is activated by Hageman factor and its fragments is important in initiating aggregation through release of arachidonic acid. Conceivably bradykinin could act on either the platelet, on the endothelium, or both. The objective of the present study is to examine the effect of bradykinin on platelet aggregation.

Methods: Into plastic syringes thirty milliliters of blood was drawn from human volunteers. The blood was transferred to a plastic tube containing 300 µl of a 0.4% solution of sodium citrate. The tube was spun at 800 g's for 3.5 minutes and the platelet rich supernantant collected. Platelet counts were performed by phase microscopy. The platelet rich plasma was adjusted to a count of 300,000 plts/mm^3 with platelet poor plasma. The platelet rich plasma was incubated under CO_2 and air to maintain the pH in the physiologic range. A dual channel Payton Aggregometer with a 2 channel recorder was used to follow platelet aggregation by light transmission. Temperature was set at 37°C and the platelet rich plasma was incubated for 2 minutes at 37°C prior to initiation of the study. The plasma was continuously stirred at 1200 rpm. Concentrations of adenosine diphosphate (ADP) $2x10^{-4}$ to $2x10^{-6}$ M in the incubation cuvette were used to induce aggregation. Concentrations of bradykinin of 30 to 500 ng were used in the following experiments.

Results:

1. Bradykinin in .01 N HCl was added to platelet-rich plasma. Bradykinin did not induce aggregation. Bradykinin did not interfere with ADP-induced platelet aggregation. The .01N HCl vehicle was added for control purposes and did not alter the findings.
2. Bradykinin did not alter the decrease in platelet aggregation following a fifteen minute incubation of platelets with aspirin.
3. Experiments using the kininase inhibitor 1,10 phenanthroline (.05 M in 25% ETOH) were performed. At a final concentration of 1,10 phenanthroline of 10^{-3} M, phenanthroline does not block ADP-induced platelet aggregation but should inhibit kininases. Bradykinin degradation should be diminished if not abolished. Even in the presence of this kininase inhibitor bradykinin did not result in platelet aggregation. The ADP-induced aggregation occurred in the usual manner.

Proposed Course of Project: The results suggest no direct effect of bradykinin on platelet aggregation. It may be that the kinin-induced release of arachidonic acid from the vascular wall may be a mode of action through which bradykinin may alter platelet aggregation. The current model while useful for testing the effect of bradykinin on platelets does not permit investigation of the interactions with the vascular wall. As such the current model will not be further employed in its present form.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01924-01 HE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Studies of Preparations of Microvessels from Rat Brains and Meninges.

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

P.I.:	Michael Fordis, M.D.	Staff Associate	NHLBI HE
OTHER:	Harry R. Keiser, M.D.	Deputy Chief, Hypertension-Endocrine Branch	NHLBI HE

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics Section

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.15	0.15	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A technique to isolate cerebral microvessels was examined. The technique permits one to isolate small arterioles, venules, and capillaries from the cerebral cortex. In addition a new technique to isolate the muscular arteries from the cortical surface was developed. A pilot study demonstrated that adequate levels of tritiated lysine can be incorporated into vascular proteins to study protein synthesis.

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Objective: Investigation of the biochemistry of the cerebral vasculature has been complicated by the difficulty in isolation of vascular elements. Recently, techniques have become available for isolation of cerebral microvessels. The purpose of the present investigation is to evaluate the technique of microvessel isolation.

Methods: Rats are killed by decapitation and the brains removed from the skulls. The cerebral cortical hemispheres are isolated and the lateral ventricles entered. Removal of the choroid plexus avoids contamination of the cerebral microvessels by a non-cortical vascular bed. The pia is gently stripped from the surface of the hemisphere. The cortex is then opened and the striatum and white matter removed. Any remaining elements of diencephalon or more caudal structures are removed at that time. The cortical pieces are then transferred to a Dounce-type tissue homogenizer and homogenized following two-fold dilution with saline. The homogenate is then poured through a 196 μm silk screen and washed with 10 ml saline. The collected material is dislodged with saline ejected through a 26 gauge needle from a plastic syringe. The homogenate is then transferred back to the homogenizer and re-homogenized. The material is then filtered serially through a 105 μm, a 75 μm, and a second 75 μm screen. The material on the three screens is washed and then dislodged. The microvessel preparation is examined under a dissecting microscope. If microvessels are present, the mixture is rehomogenized, collected on 105 μm and 75 μm screens, washed and rehomogenized. The final mixture is collected on a 49 μm screen.

In a second part of the procedure a method to remove the pia and the muscular arteries intact has been developed. This procedure will permit examination of the larger resistance vessels in addition to the smaller arterioles.

Results: Homogenization of rat cortex and selective filtering through silk screens permitted the isolation of cerebral microvessels. The microvessel preparation is a mixture of capillaries, vessels, small arterioles. Further separation of the mixture with additional filtration or with high speed centrifugation did not prove practical.

Separation of muscular arteries in the pia from the cortex proved practical. Small amounts of gray matter remained attached to vessels and pia. The present technique permits cleaning of vessels in a manner that is at least the equal of previous techniques.

Plan: Protein synthesis in the vascular wall may be dependent on the sympathetic nervous system. Some studies demonstrate increases in vascular protein synthesis that precede the onset of hypertension in certain hypertensive strains of rats compared to controls. The above technique will be used to examine the relationship of protein synthesis (in the small arteries and in the muscular arteries) to the development of hypertension in rats. The role of the sympathetic nervous system will be evaluated through pharmacologic manipulation with antihypertensives, some of which are sympatholytic and others which are not.

Other studies evaluating the presence of components of the renin angiotensin system in cerebral microvessels and in cerebral muscular arteries will be conducted.

Pilot Study: Experiments were performed to assess the ease with which protein synthesis can be measured in the cerebral muscular arteries of the rat in vivo. Both Wistar/Kyoto (WK) and spontaneously hypertensive rats (SHR) were used. Rats weighed 150 to 200 gm. Tail veins were catheterized and rats were injected with 0.4 μCi/100 gm body wt. of tritiated lysine (72.13 Ci/mmole). Injected volumes were 0.1 ml/100 gm. All dilutions were performed with physiological saline. Incubations were two hours as previously published studies demonstrated maximal incorporation of tritiated lysine at about 2 hours after intravenous injection. After two hours the animals were anesthetized with Brevital and a thoracotomy was performed. A nineteen gauge needle was used to enter the left ventricular cavity. The right atrium was incised. The animal was then perfused with saline to wash any remaining tritiated lysine in the blood out of the vessels. The brain was removed and the pial vessels were isolated. The vessels were homogenized and the incorporated lysine counted on a scintillation counter.

Scintillation Counter Results:

Animal	Total Counts Incorporated
WK	67 cpm
WK	68 cpm
SHR	69 cpm

The experiment was repeated using 40 μCi/100 gm body weight.

Animal	Total Counts (cpm)	Weight of pia (gm)	cpm/.1 gm
WK	6816	.12944	5267
SHR	6598	.15460	4268

Plan: If .4 μCi/gm of lysine is injected, high levels of radioactivity are incorporated into the pial vessels. These levels are acceptable for investigations of protein synthesis.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01925-01 HE

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Study of Leupeptin as an Inhibitor of Kallikrein

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

P.I.:	Michael Fordis, M.D.	Staff Associate	NHLBI HE
OTHER:	Harry R. Keiser, M.D.	Deputy Chief, Hypertension-Endocrine Branch	NHLBI HE

COOPERATING UNITS (if any)

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Experimental Therapeutics Section

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, 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)

Leupeptin, a modified tripeptide, can inhibit kallikrein as well as other proteases. The purpose of the current investigation was to evaluate the potency of leupeptin as a kallikrein inhibitor. The TAME esterase assay was used to measure kallikrein activity in the presence and absence of leupeptin. Dixon plots were made and Ki calculated. The Ki is on the order of 10^{-5} M which is 2 to 4 orders of magnitude less potent than other kallikrein inhibitors. This makes leupeptin much less attractive for current studies.

Objective: Leupeptin, a modified tripeptide, is a protease inhibitor with action against plasmin, trypsin, papain, cathepsins, renin, and reportedly kallikrein. When applied to skin it has been shown to block the pain and blister formation from recent thermal trauma. Pain and blister formation in a burn are initiated in part by kinins. A potent kinin synthesis blocker would be of interest. Leupeptin has relatively low toxicity and would have the advantage over other kallikrein blockers that it is small enough to be nonimmunogenic. Furthermore it can enter the intracellular space and remain active as has been demonstrated in studies of its use in muscular dystrophy. The purpose of the current experiment is to determine the relative effectiveness of leupeptin as a kallikrein inhibitor by measuring the Ki (inhibitor constant).

Methods: Kallikrein activity was measured via the radiochemical esterolytic method developed by Beaven. Purified α-N-tosyl-L-arginine-[^{3}H]-methyl-ester (TAME) was used as the substrate. Purified kallikrein from human urine was the enzyme. The activity of kallikrein was measured at 3 different concentrations of substrate. Similarly, at three different substrate concentrations, the activity of standard kallikrein was measured in the presence of leupeptin at final concentrations of $3x10^{-6}$ M to $3x10^{-5}$ M. The velocity of reaction was proportional to the net radioactivity above background.

Results:

1/velocity

µl TAME / Leupeptin ml	15	20	25
0	.0244	.0160	.0140
$3x10^{-6}$	.0257	.0188	.0152
$6x10^{-6}$	.0284	.0193	.0154
$15x10^{-6}$	.0300	.0257	.0202
$30x10^{-6}$	.0442	.0251	.0259

A Dixon plot was constructed and the Ki read off the graph. Use of three substrate concentrations allowed us to cross-check the calculated Ki.

Ki's of 1.8×10^{-5} M, 3.2×10^{-5} M were calculated.

The true Ki may be the average value of 2.5×10^{-5} M.

Compared to other kallikrein inhibitors, leupeptin is less potent an antagonist by 2 to 4 orders of magnitude. While it is not specific for kalli-

Inhibitor	M.W.	Ki
SBTI	22,000	10^{-9}
BPTI	6,500	10^{-7}
Potato	32,000	10^{-7}
Leupeptin	463	10^{-5}

krein it does have the advantages that it is small, relatively nonimmunogenic, and can gain access to intracellular spaces. For current work a much more potent kallikrein anatagonist with greater specificity would be desirable.

Proposed Course: No further work is planned for this project.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01926-01 HE

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

Comparison of a radioimmunoassay and radiochemical assay for urinary kallikrein

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

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	Harry Keiser, M.D.	Deputy Chief, HE Branch	NHLBI
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.50	0.50	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A radioimmunoassay and a radiochemical assay for urinary kallikrein were compared in normal volunteers receiving different levels of dietary salt and treatment with fludrocortisone. Overall, both assays gave similar results but the radioimmunoassay appeared to measure both prokallikrein and free kallikrein.

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PHS-6040
(Rev. 10-76)

Objectives: Our interest in kallikreins stems from their function as liberators of vasodilator kinins and from observations that excretion of urinary kallikrein was reduced in hypertensive patients. We have utilized a radiochemical assay for urinary kallikrein after validation of it by demonstration that results were equivalent to those obtained with bioassay. Following reports that results with radioimmunoassay diverge in some circumstances from those obtained with the radiochemical assay, we initiated a comparison of both methods. Because of the possibility that the radioimmunoassay might detect inactive as well as active forms of kallikrein, we studied the inactive form, prokallikrein, as well as free kallikrein.

Methods: Eleven normal volunteers were studied during successive one week periods during which they received 100 meq., 9 meq., and 259 meq. of dietary salt daily. Twenty-four hour urine samples during the last two days of each period revealed salt excretion equivalent to daily intake. Nine subjects underwent two weeks of intake of 100 meq. of salt daily and were given fludrocortisone 0.6 mg./daily during the second week.

Twenty-four hour urine samples were assayed for prokallikrein and free kallikrein using the radiochemical esterolytic method of Beavan et al. Kallikrein was determined both in untreated gel-filtered samples and after trypsin activation which converts inactive prokallikrein to active or free kallikrein; the difference between kallikrein levels in unactivated and activated samples was designated as prokallikrein, whereas kallikrein in unactivated samples was designated as free kallikrein.

Radioimmunoassay of gel-filtered urine samples was performed by a method developed in collaboration with the Laboratory of Physiological Chemistry. Antibody was prepared by Dr. Jack Pierce by inoculation of sheep with human urinary kallikrein.

Results: The radioimmunoassay and the radiochemical assay overall gave similar results. Urinary kallikrein rose with restrictioin of dietary salt and with administration of fludrocortisone. Both free kallikrein and prokallikrein increased with stimulation, but increases of free kallikrein were proportionally greater. One subject showed exceptionally low levels of free kallikrein on all three diets, but levels could be increased with fludrocortisone; prokallikrein was normal in amount and normally responsive to stimulation; in this subject, levels of kallikrein by radioimmunoassay were only slightly decreased suggesting that the radioimmunoassay measures prokallikrein as well as free kallikrein.

MEAN DAILY EXCRETION OF URINARY KALLIKREIN ON THE LAST DAY OF EACH PERIOD

ASSAY	DIET (meq.Na.) 109	9	259	n	FLUDRO- CONTROL	CORTISONE	n
RIA (mg/day)	197*	405	181*	7	199	474*	7
ESTERASE (T.U./day)							
FREE KALLIKREIN	10.7*	18.2	7.1*	11	6.6	16.0*	9
PROKALLIKREIN	5.1	7.1	4.5	11	4.5	7.0	9

*$p < 0.05$ for comparison with a 9 meq diet or control for fludrocortisone

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Significance of findings: The radiochemical assay and the radioimmunoassay for urinary kallikrein gave similar results, with the exception that the radioimmunoassay appeared to measure prokallikrein as well as free kallikrein. Low levels of free urinary kallikrein appear to be due to decreased conversion of prokallikrein to free kallikrein.

Proposed Course of Project: Additional subjects with low urinary kallikrein will be studied. Studies of salivary kallikrein will be performed.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01927-01 HE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
THE ORIGIN AND SIGNIFICANCE OF GLANDULAR KALLIKREIN IN RAT PLASMA

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

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	John J. Pisano, Ph.D.	Chief, Physiological Chemistry Section	CH NHLBI
	Harry R. Keiser, M.D.	Deputy Chief, Hypertension-Endocrine Br.	HE NHLBI

COOPERATING UNITS (if any)
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LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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)

Summary: A sensitive, selective radioimmunoassay (RIA) for rat urinary kallikrein (RUK), capable of detecting 150 pg of antigen, has been developed. The RIA was used to examine immunoreactive glandular kallikrein in rat plasma. Normal male Sprague-Dawley rat plasma (n=6) contained 162 ± 20 ng antigen/ml ($\bar{x}$ ± SEM). Bilateral nephrectomies were performed on 5 rats while 6 underwent sham operations. Analysis of the plasmas after 44 hrs. showed that sham plasmas contained 179 ± 4.5 ng antigen/ml while nephrectomized plasmas contained 618 ± 97 ng/ml ($p < .001$). Gel filtration of pooled plasmas revealed 3 peaks of antigen (Ag) in both sham and nephrectomized plasmas. For both sham and nephrectomized plasmas, peak 3 showed parallel cross-reactivity to RUK while peaks 1 and 2 did not. Ag contents of the first two peaks were similar in sham and nephrectomized plasmas while the Ag content of the third peak increased about 9-fold in nephrectomized plasmas.

PHS-6040
(Rev. 10-76)

Objectives: 1) To establish a sensitive, specific radioimmunoassay (RIA) for rat urinary kallikrein (RUK). 2) To characterize immunoreactive antigen in rat plasma and to determine its origin(s) and physiological significance.

Methods: Antibody to RUK raised in sheep (Project No. NHLI-109) was used to set up the RIA. Labelled antigen is produced via a Chloramine T iodination procedure. The labelled protein is separated from free iodide by two consecutive gel filtrations on Sephadex G-75. Antigen-antibody complex is separated from free antigen with double antibody precipitation in the presence of sheep IgG.

Major Findings: Using antibody to RUK at a final dilution of $^1/_{400,000}$ it is possible to detect 150 pg of antigen. A variety of possible antigens have been studied. Trypsin, pepsin, bradykinin, lysyl-bradykinin, angiotensin I and rat urinary esterase A all showed no significant cross-reaction while pure rat submandibular kallikrein showed identity to RUK. Rat urine showed parallel cross-reactivity to the standard curve.

Several materials may interfere non-specifically with antigen-antibody binding at high concentrations and must be used in appropriate dilutions to avoid this problem. Heparin, polybrene , sodium azide, high levels of salt, and the anticoagulant, ACD, can all decrease antigen-antibody binding. The proteinase inhibitor, aprotinin, also can interfere, presumably competing with the antibody for binding RUK.

Intra- and inter-assay coefficients of variation (n = 6) are 2% and 5%, respectively. The recovery of purified RUK added to rat plasma (n = 6) was 100 ± 3% ($\bar{x}$ ± SEM). RUK added to urine, however, (n = 11) was only recovered at a level of 80 ± 3%.

Immunoreactive antigen was detected in normal rat plasmas (n = 6) at a level of 162 ± 20 ng/ml ($\bar{x}$ ± SEM). Bilateral nephrectomies were performed on 5 rats to see if the kidney is a major source of the plasma antigen. After 44 hours, plasmas from sham operated animals (n = 6) contained 179 ± 4.5 ng antigen/ml while that from nephrectomized animals contained 618 ± 97 ng/ml ($p<.001$)

The antigen in plasma has not consistently shown paralled cross-reactivity to the standard curve, leading to slight problems of quantitation. In order to verify the effects of nephrectomy and to further characterize the plasma antigen, pooled plasma from sham and nephrectomized rats was gel-filtered on Bio-Gel A-0.5m. The ultraviolet absorption (A_{280}) profiles for sham and nephrectomized plasmas were identical and three peaks of immunoreactive antigen were seen in each of the pooled plasmas. Peaks 1 and 2 (apparent M wts. $\geq$590,000 and 160,000, respectively) cross-reacted in a non-paralled fashion and appeared to have similar antigenic contents in plasmas from both sham and nephrectomized rats.

Peak 3 (apparent M wt ~85,000) was consistently paralled to the standard curve in both pooled plasmas. In plasma from sham-operated rats, however, this peak contained 116 ng antigen while in plasma from nephrectomized rats it contained 1075 ng. Purified RUK applied to the column had an elution volume equivalent to that of peak 3. A preliminary study showed that peak 3 had no free ^{3}H-TAME esterase activity but was extremely active after treatment with small amounts of trypsin.

These results indicate that peak 3 may represent prokallikrein or a kallikrein-inhibitor complex. The apparent molecular weight of 85,000 daltons does not correspond to the usual molecular weight of glandular kallikrein (33,000-35,000) and needs to be studied further. The kidney obviously plays a major role in the metabolism and/or clearance of glandular kallikrein in plasma.

Proposed Course: Gel filtration fractions will be examined in much greater detail to characterize the various peaks of antigen. Esterase and inhibitor studies will be performed as well as experiments to try and estimate the molecular weight of peak 3 by alternative methods. Studies are also under way to determine the effects of the other major sources of glandular kallikrein, namely pancreas and the salivary glands. A comparison of normal and bladder urine will be made to determine if rat urinary esterase A is a bacterial artefact. Rat/renal lymph samples will also be examined for glandular kallikrein with the RIA. The low recovery of pure RUK added to urine will be studied further and considered in relation to the radiometric TAME assay to see how it affects correlations between the assays.

Publications: Lawton, W. J., Proud, D., Pierce, J. V., Pisano, J. J., and Keiser, H. R.: Bilateral nephrectomy raises the level of immunoreactive glandular kallikrein in rat plasma (Abstract). Clin. Res. 27(2):42, 1979.

ANNUAL REPORT
SECTION ON BIOCHEMICAL PHARMACOLOGY
HYPERTENSION-ENDOCRINE BRANCH
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1978 through September 30, 1979

The research of this section covers a rather wide range of topics related to regulation of neuronal systems, and the cellular biochemical response to neurotransmitters. We are also interested in how neuronal systems interact with each other and finally how these systems have an impact on the physiological regulation of blood pressure.

I. Characterization and Regulation of Neurotransmitter Biosynthetic Enzymes

There are several major neuronal networks in the central nervous system for which biogenic amines serve as neurotransmitters. These amines are dopamine, norpinephrine, epinephrine and serotonin. The catecholamines are derived from tyrosine whereas tryptophan serves as a precursor for serotonin. Since there is considerable evidence that the synthesis of the neurotransmitters is coupled to functional activity of individual neurons, one of our primary objectives has been to characterize the biosynthetic enzymes in each of these transmitter pathways. Prior work in our laboratory had demonstrated that tyrosine hydroxylase could exist in different activity states and that the activation could be accomplished by a cAMP dependent protein phosphorylation. Furthermore, it appeared that the major impact of phosphorylation was a decrease in the sensitivity of the enzyme to feedback inhibition. Recent experiments have demonstrated that a Ca^{++} stimulated protein kinase is as effective as the cAMP dependent protein kinase in catalyzing the phosphorylation and activation of tyrosine hydroxylase. These studies have also demonstrated for the first time that phosphate is incorporated directly into the major subunit of tyrosine hydroxylase. Concurrent studies on the molecular parameters of tyrosine hydroxylase suggest that this protein may be a tetramer of 60,000 molecular weight subunits. Studies from ours and other laboratories suggest that the incorporation of a single phosphate group per subunit is sufficient to result in activation.

The degree and kinetic nature of the activation of tyrosine hydroxylase by protein phosphorylation has recently been the subject of controversy. Our initial studies on this reaction in 1975 and 1976 indicated simply that in crude striatal extracts, the K_m for tetrahydrobiopterin was reduced. Later studies indicated that the major effect was on K_i for dopamine. Other laboratories have shown using highly purified preparations of tyrosine hydroxylase that phosphorylation resulted in a significant increase in V_{max} with minimal changes in K_m for the hydroxylase cofactor. Recent studies in our laboratory have shown that the pH of the reaction mixture greatly influences the degree of activation. With semi-purified enzyme preparations we find that phosphorylation causes a slight alkaline shift in pH optimum (5.8 to 6.0), but also broadens the activity curve on the alkaline side, so that phosphorylation effects are seen much more dramatically in range of pH 6.3 to 7.0 even though activity is suboptimal.

Understanding the putative physiological role for this regulatory mechanism is one of our major objectives. During the past year we have expanded and extended our studies on the mechanism of neuroleptic drugs. Dopamine receptor

blockers cause a rapid activation of tyrosine hydroxylase in the striatum. Like the in vitro phosphorylation, dopamine receptor blockade causes the pH optimum of tyrosine hydroxylase to broaden on the alkaline side with the maximum degree of activation seen at about pH 6.3, although optimum activity was observed between pH 5.8 and 6.0. We previously reported that chronic treatment of rats with haloperidol result in a deactivation of tyrosine hydroxylase rather than an activation. In the past year this concept was challenged by other laboratories and upon reinvestigation we found that the effect that we were observing was not apparent when reaction mixtures were maintained at pH 5.8 (optimum), but were readily observed at pH 6.3. Further investigation of the effect of long-term treatment of animals with haloperidol indicated that when the striatal enzyme from these animals was partially purified that the deactivation phenomenon was primarily a decrease in the K_i value for dopamine. This suggests that tyrosine hydroxylase becomes more sensitive to end-product inhibition after animals receive a prolonged treatment with a neuroleptic agent.

Based on an analogous concept of receptor mediated control of tyrosine hydroxylase we predicted that administration of the alpha agonist clonidine would result in a deactivation of tyrosine hydroxylase in brain regions rich in noradrenergic nerve terminals. Control experiments measuring MOPEG sulfate, a major norepinephrine metabolite indicate that indeed synthesis of norepinephrine was markedly reduced. Unfortunately we have yet to demonstrate a deactivation of tyrosine hydroxylase. This failure may be explained by our recent pH studies and this problem will be reinvestigated in the coming year.

Our research findings on this important system comfirm the concept that the synthesis of catecholamines is regulated by the activity of tyrosine hydroxylase. This enzyme system appears to be subject to rapid regulation in vivo by a protein phosphorylation reaction. The 3 major factors that determine activity appear to be the degree of phosphorylation, end-product concentration, and hydroxylase cofactor concentration.

In the previous year we reported modification of methodology for the measurement of hydroxylase cofactor in discreet brain regions and cerebrospinal fluid. We have found that the concentration of hydroxylase cofactor correlated fairly with the tissue content of tyrosine and tryptophan hydroxylase in most brain regions. The concentration would, however, appear to be subsaturating for the hydroxylases in most regions. An interesting sidelight to this investigation was the finding that tissues related to neuroendocrine function contained levels of hydroxylase cofactor that were disproportionately high when compared to their hydroxylase content. We suggested that tetrahydrobiopterin may play an unknown neuroendocrine role. It is of interest that another laboratory recently identified the prolactin inhibitory factor of the pineal gland as tetrahydrobiopterin. We have also examined the hydroxylase cofactor content of the CSF from a large number of control subjects and patients with neurological diseases. Parkinsonian patients exhibited hydroxylase cofactor content of the CSF which was about 50% that of age-matched controls. This is consistent with their known loss of dopamine synthesizing capacity. The suggestion that there is a decreasing dopamine function in the brain with age is also born-out by our observed inverse correlation of CSF hydroxylase cofactor with age. We also recently reported a mother and 4 daughters, all have extremely low levels of hydroxylase cofactor in their CSF and all significantly affected by familiar dystonia. Overall, these studies

are consistent with the above mentioned concept for the regulation of catecholamine synthesis, i.e., the extremely important role of hydroxylase cofactor in dopaminergic function.

Serotonin is another important neurotransmitter of brain and its synthesis is regulated by the enzyme tryptophan hydroxylase. During the past year our understanding of this enzyme has increased significantly. While tryptophan hydroxylase is similar to tyrosine hydroxylase in many ways it is a distinct enzyme and is remarkably less stable. For this reason it has not been possible to obtain purified preparations. In our previous reports we demonstrated that tryptophan hydroxylase could also be kinetically activated by a protein phosphorylation reaction. The protein kinase responsible for this activation was cAMP independent but apparently totally dependent upon Ca^{++}. A protein kinase that is activated by a protease and is also partially dependent upon Ca^{++} has been isolated from brain. It is thought that this protein kinase may be related to the enzyme responsible for the activation of tryptophan hydroxylase. In an effort to understand this hydroxylating system more fully we have compared the regulatory properties of the tryptophan hydroxylase from malignant mouse mast cell with that from rat brain. The enzyme from mast cells has a molecular weight of 144,000 compared to 220,000 from the enzyme from rat brain. The latter enzyme can be activated by Ca^{++}, sodium dodecyl sulfate, trypsin, phospholipids and protein phosphorylation, whereas the mast cell enzyme is inhibited by these treatments. A recent report suggests that the brain enzyme acquires properties similar to that of mast cells if it is first activated by proteolysis. It is entirely possible that the enzyme in the malignant mast cell has already been subjected to intracellular proteolysis. In order to establish conditions for the isolation tryptophan hydroxylase the nature of the inactivation has been studied. Maintenance of the enzyme solution under strict anaerobic conditions dramatically stabilizes the activity. If a solution of tryptophan hydroxylase has been deactivated by exposure to air, most of the activity can be recovered by incubation with Fe^{++}, a mercaptan and inorganic sulfide, under anaerobic conditions. This suggests the possibility that tryptophan hydroxylase is an iron sulfur protein. With this increased knowledge we plan in the coming year to purify brain tryptophan hydroxylase.

II. Receptor Mechanisms and Cyclic Nucleotide Metabolism

Neurotransmitters appear to have their initial impact on target cells through an alteration in cyclic nucleotide metabolism. While the exact coupling mechanism between the receptor and cyclic nucleotide metabolism is not known, it appears Ca^{++} and the Ca^{++} binding protein, calmodulin, are important. During the past year an increasing number of studies in our laboratory have focussed on the role of calmodulin and the nature of the phosphodiesterases. In bovine pineal gland a new form of this enzyme has been observed. While the specific function of this form of phosphodiesterase is unknown, its unusual properties made it an interesting enzyme for study. The enzyme is relatively resistant to heat inactivation, it is specific for cAMP and it appears not to be influenced by Ca^{++} and calmodulin although it has a relatively high K_m for its substrate. It is possible that this enzyme is derived from the normal phosphodiesterase of the pineal which is regulated by calmodulin.

A cAMP specific phosphodiesterase has also been identified in a malignant

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tumor (P815) of murine mast cells. This enzyme occurs in a single molecular and kinetic form (low K_m), and is apparently not dependent on calcium and calmodulin for optimum activity. The enzyme is inhibited by cGMP and the sulfated mucopolysaccharide, heparin. The mast cell phosphodiesterase displays anomalous behavior on gel filtration and sucrose density gradient centrifugation. The Stokes radius was determined by gel filtration to be 54.4A and the $S_{20,w}$ was determined by gradient centrifugation to be 2.73S. Using these values a molecular weight of 61,000 and a frictional ratio of 1.93 were calculated. This mast cell phosphodiesterase is apparently an asymmetric enzyme molecule with novel molecular and regulatory properties.

In brain however the major form of phosphodiesterase appears to be that which has been widely studied and whose activity is regulated by Ca^{++} and calmodulin. This enzyme is related to receptor function and is apparently part of a self limitation mechanism for receptor activation. The striatal portion of brain contains a large number of dopaminergic nerve terminals and cells that contain dopamine receptors. We have shown that in striatal slices dopamine receptor activation results in the translocation of calmodulin from membrane bound stores to the cytoplasm. This process results in the association of a significant amount of calmodulin with the larger molecular weight fraction (presumably phosphodiesterase) and classical receptor antagonists blocked by this reaction. Prior nigra-striatal transection did not affect the observed phenomenon suggesting that the release of calmodulin and the activation of phosphodiesterase was occuring primarily in post-synaptic cells. In a separate but related study we found that incubation of striatal slices with morphine or enkephalin resulted in a similar release of calmodulin and activation of phosphodiesterase. This effect could be blocked by both opiate and dopamine receptor blockers and by prior transection of the nigra-striatal pathways. These observations would suggest that dopamine nerve terminals may contain opiate receptors that serve to modulate the dopamine neurons.

Although the initial isolation and characterization of calmodulin was based on its ability to activate a calmodulin-deficient brain phosphodiesterase, the assay based on this phenomenon had been difficult to qualify. Early studies indicated difficulty in preparing antibodies. Following the coupling of pure calmodulin to hemocyanin, antisera to calmodulin were produced in rabbits. With antibodies, a specific and sensitive enzyme linked immunosorbent assay has been developed which has been a significant asset in the above studies.

We have also studied the regulation of cyclic nucleotide levels in the pineal gland. In this organ it is known that β-receptors that are activated by norepinephrine are closely coupled to an adenylate cyclase. Thus, stimulation of the β-receptor *in vivo* or in organ culture leads to a rapid increase in cAMP content. Conversely β-receptor blockade causes a rapid fall in intracellular cAMP content. We have found that gland which have been stimulated by darkness *in vivo* or by a β agonist *in vitro* responds to β-blockade by a rapid and dramatic increase in cytoplasmic calmodulin and an associated activation of phosphodiesterase. These observations provide a molecular explanation for the extreme changes in cyclic nucleotide that take place in this organ and gives some insight into the dynamics of cyclic nucleotide metabolism.

III. Interaction of Neurotransmitters with Target Cells

The pineal gland offers an unusual opportunity to study the interaction of a neurotransmitter system with its target, the pinealocytes, which contain β-receptors and are innervated almost exclusively by noreadrenergic sympathetic fibers. The well known response to stimulation of pineal β-receptor is an apparent induction of the enzyme serotonin-N-acetyltransferase. This enzyme regulates the rate of melatonin synthesis by the pineal gland. Work in previous years had explored the molecular events that occur between the activation of the receptor and the appearance of increased enzyme activity. While the increments in cAMP and protein kinase activity led to an increased phosphorylation of a specific nuclear protein, there appears to be no increases in overall or specific RNA synthesis. Studies on amino acid nicroporation did not reveal the selective synthesis of any particular protein band and our conclusion was that the increased enzyme activity was due to a post-translational modification of the enzyme or that the in vivo rate of enzyme degradation was changing.

Since induction of an enzyme usually involves increase in RNA polymerase activity and increases in ornithine decarboxylase often associated with changes in polymerase activity we examined ornithine decarboxylase during the induction process. Only minor changes in ornithine decarboxylase activity were observed during the induction phenomenon.

Since it is known that nerve impulses generated in the eye lead to modulation of the neuronal imput to the pineal gland we probed this neuronal pathway with pharmacological agents using N-acetyltransferase (NAT) as the indicator of neuronal activity arriving from the superior cervical ganglion. Rats exposed to the dark have high levels of NAT activity which falls rapidly when exposed to light. We found that the α-receptor blocker phenoxybenzamine could prolong the dark levels of NAT activity after the animals were exposed to light. Conversely administration of the agonist clonidine resulted in a rapid fall in enzyme activity in animals continually exposed to darkness. This latter response could not be blocked by yohimbine. We therefore conclude that there must be at least 2 α type receptors in the central nervous system modulating the neuronal regulation of NAT.

Another series of experiments focussed on the possible existence of a similar melatonin producing system in the retina. This tissue contained significant amounts of NAT, the activity of which appeared to increase during the dark hours.

IV. Chemoreceptor Regulation

Studies in prior years established that rats respond to hypoxia with a depletion of dopamine from the carotid body and a long-term increase in tyrosine hydroxylase activity. We have recently found that stimulation of muscarinic receptors by methacholine will mimic the hypoxic response in that the rate of dopamine released in the carotid body is increased without changing its immediate turnover rate. This is consistent with the fall in dopamine content observed. Neither the carotid sinus nerve nor the sympathetic nerves appear to participate in the normal dopamine release. Of interest is the observation that carbohydrate active steroids appear to block dopamine metabolism in the carotid body. Studies are continuing on the molecular mechanisms which are utilized in the carotid body

for adaptation to environmental changes.

V. Studies on the Spontaneously Hypertensive Rats (SHR)

Our laboratory has for several years investigated a number of neuronal systems that may contribute to the development of increased blood pressure in the SHR. Since a large background of information suggests that activation of the central α receptors tends to modify the hypertension in SHR, we undertook an investigation of regional central α-receptors by ligand-binding techniques with the antagonist WB-4101 and the agonist, clonidine. While these experiments are still in the early stages it is of interest that while no differences in WB-4101 binding are observed, there is a significant decrease in clonidine binding in the hippocampus, locus coeruleus and A_2 region of the SHR. These observations are consistent with our general hypothesis of central blood pressure reguation, but considerably more data are needed.

Another central neuronal system of particular interest in blood pressure regulation is epinephrine-containing cell group. We have confirmed earlier observations from Axelrod's laboratory that the activity of phenylethanolamine-N-methyltransferase (PNMT) is slightly increased in the A_2 region of the brain. This enzyme which has been studied largely in the adrenal medulla appears to be partially under control of the pituitary adrenal axis. Hypophysectomy results in a decrease in the activity of PNMT in the A_2 region and this effect is reversed by dexamethosone. While the evidence to date that adrenergic neurons are involved in blood pressure regulation is not well substantiated it is of interest that the activity of these neurons may be altered in the SHR. Recently considerable attention has been addressed to 3-deaazadenosine, a compound that inhibits methylation reactions by causing an accumulation of S-adenosylhomocysteine. With the possible overactivity of adrenergic neurons in SHR, it was of interest to determine the effect of this compound on blood pressure in the SHR. 3-deaazadenosine caused a rapid and marked fall in blood pressure. The rapidity of the fall suggested that this was a direct-acting hypotensive agent that was possibly a vasodilator. Comparison of the response to that of hydralazine or adenosine 2 known vasodilators in presence and absence of ganglionic blockade was consistent with such a concept. This observation may be of little therapeutic value in itself, but it does suggest a new approach to the development of antihypertensive agents.

Recent studies have shown that the adrenal medulla contains significant amounts of enkephalin-like peptides. These peptides have been partially purified and characterized by Costa and his colleagues. One of these peptides whose structure is still unknown has been shown to have a powerful depressor effect in both normotensive rats and SHR. We are continuing investigation of the potential role of peptide substances from the adrenal in modulating blood pressure.

Along a somewhat different line, we observed that the incidence of stroke in the stroke-prone substrain of the SHR was very low in our colonies as compared to the Japanese colonies. We therefore initiated a crossover study with the rats and diets from both the U.S. and Japan. After 10 months the incidence of stroke in either group of rats consuming the Japanese diet was over 50% whereas no apparent stroke was observed in the stroke-prone rats consuming the NIH diet. No differences in growth rate were observed only slight differences

in the degree of hypertension. Analysis of the diets revealed only that the NIH diet contained 24% protein whereas the Japanese diet contained 17% protein. The importance of this work is the demonstration that an interaction of environmental (diet) and genetic factors are responsible for the high incidence stroke. Recent epidemiological studies in Japan have revealed a very similar pattern for the incidence of stroke in man.

VI. Miscellaneous

Morphine and Membrane Phosphorylation

Little is known of the specific cellular systems involved in morphine tolerance and dependence. A number of lines of evidence indicate protein phosphorylation systems may be altered. Our studies have shown that the phosphorylation of synaptic plasma membrane, from the brains of mice that are tolerant to and dependent upon morphine is stimulated to a greater extent by calcium and calmodulin than are control membranes. The stimulation is seen only in specific protein bands. A similar pattern is observed for the incorporation of $^{32}PO_4$ into synaptosomal proteins. Of particular interest was observed increase in Ca^{++} dependence on the conversion of tyrosine to DOPA in synaptosomes from morphine treated animals. All these findings would be consistent with a depletion of calmodulin from synaptic membranes during morphine treatment.

Substance P

During the past year we have initiated studies on the role of substance P as a neurotransmitter in the central nervous system. While our initial hypothesis suggested that Substance P may coinhabit with serotonin in neurons, our results to date have not confirmed this concept. Of more interest however are our findings that Substance P is markedly depleted from its nerve endings following chronic treatment with a dopamine receptor blocker. Clearly it is one of the modulatory systems for dopaminergic neurons and will present a useful tool for studying neuronal interactions. We have recently found that converting enzyme inhibitors when administered centrally also raise the concentration of Substance P and its relation to blood pressure regulation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01843-06 HE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Mechanisms of Storage Uptake and Release of Norepinephrine Adrenergic Nerve Endings (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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COOPERATING UNITS (if any)
NONE

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 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)

Evidence is presented to substantiate and more precisely define the postulate that catecholamine neucoltransmitters may be released by a neuronal transport process distinct from exocytosis. This is a continuing investigation of a model synaptic system consisting of a calcium dependent release of ^{3}H-norepinephrine (^{3}H-NE) from rat heart slices incubated in a Na^{+} deficient medium. It appears that a calcium dependent process directs norepinephrine loaded intraneuronal binding sites to a transport site on the plasma membrane. The ^{3}H-NE appears to be passed directly from its binding site to the outward transport system. Amines therefore can be transported across an intact membrane without involving a process of exocytosis. Reserpine and Li^{+} appear to disrupt this process since they elicit a calcium independent release of deaminated metabolites.

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Objectives: To define the mechanisms of Ca^{++} dependent mobilization and release of ^{3}H-NE stored in adrenergic nerves in rat heart ventricle slices.

Methods: Rat heart ventricle slices were labelled with ^{3}H-NE In Vivo and then ventricle slices were prepared and incubated as described in earlier reports. The medium was sampled for the determination of the total radioactivity to be used for calculating the rates of release of ^{3}H-compounds. In other experiments the entire medium was saved for the separation on resin columns and the determination of total ^{3}H-amine and total ^{3}H-deaminated metabolites. In any given experiment, all tissues were either incubated for the same length of time or the incubation was terminated at various times after the start of incubation. Tissues were processed for the determination of the proportions ^{3}H-catecholamine precursor remaining as ^{3}H-amines or ^{3}H-deaminated metabolites. For the estimation of ^{3}H-amines and ^{3}H-metabolites, slices were preincubated 60 minutes, then the incubations were continued for 3 additional 70 minute and, 1, 60 minute period, making a total of 5 hours. The quantity of ^{3}H-compounds released from tissues incubated in KRBS, with or without cocaine, was designated as control-level release. Changes from the control level release of ^{3}H-compounds in KRBS, as described below, are based upon parallel runs of tissues in KRBS and in the test medium. These changes refer to percentages of the amount of ^{3}H-compounds originally present in the tissue.

Major Findings: The results of this series of experiments is summarized as follows:

1) ^{3}H-deaminated metabolites comprise 85% of the ^{3}H-compounds released from slices incubated in the krebs-bicarbonate control medium (KRBS) during the first period of incubation onward (excluding the preincubation).

2) Although this percentage of ^{3}H-deaminated metabolites might be decreased by cocaine present during the preincubation, the percent was not changed by the cocaine during the subsequent incubations.

3) The quantity of ^{3}H-amines released in the Na^{+}-deficient choline medium was greatly increased. However, the quantity of ^{3}H-deaminated metabolites was increased only slightly. The difference between the quantities of ^{3}H-compounds released in the Na^{+}-deprived and in the control tissues in parallel runs was designated as stimulated release. The similarity in the quantities of ^{3}H-deaminated metabolites released in the two media suggested that the release of these metabolites is a separate phenomenon from the stimulated release of ^{3}H-amines. As shown in earlier reports, the latter is a Ca^{++}-dependent release.

4) Cocaine blocked the stimulated release of both ^{3}H-amines and ^{3}H-deaminated metabolites. Moreover, the quantity of ^{3}H-deaminated metabolites in stimulated (Na^{+}-deprived) tissues was not decreased by the presence of cocaine. Thus, the stimulated release of ^{3}H-deaminated metabolites was the result of deamination that occurred after the release of tissue ^{3}H-amine in Na^{+}-deprived

tissues. These ^{3}H-metabolites were therefore included with the ^{3}H-amines released by the stimulation with Na^{+} deficient ($choline^{+}$) media containing calcium. The control level deamination of ^{3}H-amines occurred intraneuronally and was independent of the mobilization and release of ^{3}H-amines. It could be related to the quantity of ^{3}H-amines mobilized in the immediate vicinity of the plasma membrane. Since the availability of monamine oxidase at this point is limited, the enzyme is postulated to operate at near saturation when the quantity of tissue ^{3}H-amine is high but the rate of mobilization is slow, as in KRBS, or when the quantity of tissue ^{3}H-amine is low but the rate of mobilization is fast in Na^{+}-deprived tissues. Consequently, the control level release of ^{3}H-deaminated metabolites is independent of the rate of mobilization and release of ^{3}H-amines in the neuron incubated in any of the media. The small quantity of ^{3}H-amines among the ^{3}H-compounds released in KRBS is not changed by cocaine, and is regarded as being released by unknown pathways. These ^{3}H-amines are regarded as part of control level release. Hence, the control level release of total ^{3}H-compounds in KRBS is quantitatively equivalent to control level release of ^{3}H-compounds in Na^{+}-deficient (choline) medium. The difference between the total ^{3}H-compounds released in the two media actually represents the cocaine sensitive, stimulated release of ^{3}H-amines. This simple calculation is more accurate than the difference between ^{3}H-amines estimated for the two media. The latter method would underestimate the stimulated release of ^{3}H-amines by eliminating ^{3}H-amines which were released from the neurons but were secondarily deaminated before entering the medium.

The monoamine oxidase inhibitor, pargyline, prevents the deamination of ^{3}H-amines, and ^{3}H-amines almost exclusively represented control-level release of ^{3}H-compounds. However, pargyline inhibited the release of these amines in control and Na^{+}-deprived tissue. These amines, therefore, complicate the release curves whereas they would otherwise be deaminated and eliminated from the calculated quantity of ^{3}H-amines released by stimulation.

5) Reserpine increased the proportion of ^{3}H-compounds released as ^{3}H-deaminated metabolites in KRBS to more than 90%.

The above findings were used in experiments to show the site of mobilization and release of ^{3}H-amines in adrenergic neurons in rat ventricle slices. It has been previously reported that the Ca^{++}-dependent stimulated release of ^{3}H-amines in Na^{+}-deprived heart slices was transport mediated. The combination of reserpine and Na^{+}-deprivation does not basically change the pattern of release of ^{3}H-amines and ^{3}H-deaminated metabolites. There is an increment of ^{3}H-deaminated metabolites released, but also an increment of amines released relative to the release of these ^{3}H-compounds in the Na^{+}-deprived medium without reserpine. Thus, each releasing agent acts relatively independently.

Cocaine blocks the Ca^{++}-stimulated release of ^{3}H-amines in Na^{+}-deprived tissues by blocking transport. The combination of cocaine with the Na^{+}-deprivation and reserpine elicits the rapid release of ^{3}H-deaminated metabolites almost exclusively.

The interpretation of these findings follows:

1) The Ca^{++}-dependent mechanism draws binding sites to the carrier mechanism at the plasma membrane. Bound amine is mobilized and transported out of the nerve endings.

2) Cocaine blocks the transported release of ^3H-amine, but the trapped amine was not deaminated, indicating that the amine was not mobilized in the presence of cocaine. If it were mobilized, increased deamination would be expected.

3) Reserpine and Na^+-deficient ($choline^+$) media, with Ca^{++}, exert their respective actions independently. Some of the ^3H-amines mobilized by reserpine are transported and some are deaminated, but there is no generalized deamination of mobile ^3H-amines characterized of reserpine alone.

4) In Na^+-deprived tissues with Ca^{++} and reserpine, cocaine blocks the transported release of amines, but cocaine makes the reserpine-mobilized ^3H-NE available to be deaminated.

5) Most remaining tissue ^3H-amine remains as such regardless of the medium or releasing agents employed. Reserpine increases the proportion of ^3H-deaminated metabolites remaining in the tissue but does not apparently cause a rapid, massive release of vesicular NE, which would cause most of the tissue ^3H-compounds to be present as deaminated metabolites. Even in the presence of reserpine and cocaine, Na^+-deprived tissues, in which mobilized amines are trapped by cocaine and deaminated before release, retain more than 70% of their remaining tissue ^3H-compounds in the form of ^3H-amine. The mobilization reactions occur at or near the plasma membrane. There is no generalized mobilization of ^3H-amines which could result in rapid deamination of all remaining tissue ^3H-amines. Although the ^3H-amines are mobilized at the membrane, cocaine prevents their being mobilized at the transport sites. This appears to be related to the rapid deamination of the mobilized ^3H-amines contrasting with the slow deamination of mobilized amines at transport sites. Experiments with reserpinized tissues with pargyline and cocaine lead to a similar conclusion. Pargyline inhibits deamination, and the ^3H-amines are released more slowly than the ^3H-deaminated compounds, but the release of ^3H-amines is not blocked by cocaine. Although cocaine slightly inhibits the release of ^3H-amines in the reserpinized tissues without pargyline, the inhibitory effect is much less than the inhibition of Ca^{++}-stimulated release. Either the release of ^3H-amines by reserpinized tissues with pargyline was not transport-mediated, or cocaine did not block outward transport in media containing Na^+.

In accord with the above postulates, the T/2 of 5 minutes reported for NE in isolated splenic nerve vesicles may be excessively rapid and may not represent vesicles that are physiologically maintained in vivo. If, as postulated by others, reserpine releases vesicular amine by blocking the uptake part of a pump and leak system, having a T/2 of 5 minutes, the released ^3H-amine should all be rapidly deaminated. Our findings, however, are in disagreement with this postulate. There does not appear to be a general pre-release mobilization of ^3H-amine that could result in high concentration of free amine, which would then be deaminated. Moreover, our findings are compatible

with the postulate that mobilization and transport are intimately associated, if not one and the same mechanism in this system. Thus, transport block prevents stimulated deamination in Na^+-deprived tissues (but not control level deamination). If Ca^{++}-dependent release involved independent mobilization and transport of free amine, then blocked transport should produce more deamination, such as occurs in the presence of reserpine when cocaine has blocked transport.

The mechanism of axonal transport of NE loaded binding sites to the transport site is not blocked by colchicine or cytochalasine B (previous findings). These experiments show that the Ca^{++}-dependent release of ^3H-amines in an Na^+-deficient ($choline^+$) medium involves specific mechanisms of axonal transport of NE loaded binding sites to amine transport sites and release of amine. It appears possible that these mechanisms are involved in synaptic transmission.

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 possibly are mediated by the same mechanism.

Proposed Course of Project: It was expected that attempts would be made during the past year to inhibit adrenergic transmission with inhibitors of transport. However, these attempts have not yet been made, although it is expected that such attempts will be made.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01846-05 HE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Molecular Biology of Chemoreceptor Regulation

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

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SECTION
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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 mechanism whereby the catecholamine content of carotid bodies is regulated by physiologic or pharmacologic stimuli was studied. Hypoxia and stimulation of muscarinic receptors increases the rate of dopamine release without changing its turnover-rate. Neither the carotid sinus nor the sympathetic nerves participate in the modification of dopamine content by both types of stimuli. In contrast, carbohydrate-active steroids cause a short-term increase of dopamine content which appears to be due to blockade of dopamine metabolism.

582

PHS-6040
(Rev. 10-76)

Objectives: The carotid body is a chemoreceptor organ which senses changes in the partial pressure of arterial blood gases. 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 was 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: 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 analoges 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.

In some studies transection of the carotid sinus serve, or superior cervical ganglionectomy was performed unilaterally 5 to 7 days before the experiment.

Major Findings: Decrease of dopamine content by hypoxia. During hypoxic conditions the dopamine content in rat carotid body is decreased, due to an increased rate of release. In contrast, the norepinephrine content remains unaltered under the same conditions. Neither transection of the carotid sinus nerve nor ganglionectomy prevented the decrease in dopamine content elicited by hypoxia, therefore it is inferred that low arterial pO_2 elicits depletion of dopamine stores in carotid body independently of the above mentioned innervation.

Decrease of dopamine content by methacholine. Injection of methacholine (9 μmol/kg) decreases the dopamine content of rat carotid body without changing the norepinephrine content. This decrease in dopamine content also occurs after transection of the carotid sinus nerve or ganglionectomy. Methylatropine (21 μmol/kg) prevents the decrease of dopamine content elicited by methacholine. Since methylatropine could block also the decrease of dopamine content elicited by hypoxia it is inferred that a muscarinic receptor may play an important role in the mechanism, whereby the carotid body senses changes in arterial gases.

Increase of dopamine and norepinephrine content by carbohydrate-active steroids. Short-term or long-term administration of dexamethasone (1 mg/kg,i.p.) increases the concentrations of dopamine and norepinephrine in rat carotid body. This increase occurs also after transection of the carotid sinus nerve or ganglionectomy. Injections of cycloheximide curtail only the increase in noradrenaline content but not the increase in dopamine content.

Studies on the turnover of dopamine in carotid body revealed that the rate of elimination of DOPAC was reduced more than 50% 12 hours following the injection of dexamethasone, while the steady content of dopamine is in-

creased. From these results it was concluded that the regulation of dopamine and norepinephrine content by dexamethasone underlies two different mechanisms. The increase of dopamine content is due to a reduction in its metabolism, whereas the long-term increase of norepinephrine appears to be triggered by a process, which involves new protein synthesis.

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 chemosensory response. Our studies revealed that dopamine present in the glomus cell is the major neurotransmitter involved in the chemoreceptor response to decreased arterial pO_2.

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 drugs acting upon respiration, in particular narcotics, on the dopamine and norepinephrine content in carotid body.

2. Binding studies with cholinergic, dopamine and opiate receptor ligands to characterize nature of receptors involved in chemoreceptor regulation.

3. Studies on the effect of long-term exposure to hypoxia on the morphology and neurotransmitter content of the carotid body.

Publications:

1. Hanbauer, I. and Hellstrom, S.: The regulation of dopamine and noradrenaline in the rat carotid body and its modification by denervation and hypoxia. J. Physiol.(L) 21-34, 1978.

2. Hellstrom, S., Commissiong, J. and Hanbauer, I.: Modification of the dopamine and noradrenaline content in rat carotid body by carbohydrate-active steroids. Neuroscience (In press).

3. Hellstrom, S. and Hanbauer, I: Role of dopamine and norepinephrine in carotid body. In: Catecholamines: Basic and Clinical Frontiers (Usdin, E., Kopin, I.J. and Barchas, J.,eds.) Vol. 2, Pergamon Press, N.Y., 1979, pps. 1539-1541.

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PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Functional Role of Second Messengers in the Regulation of Neurotransmitter Receptors

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

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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)

In the supernatant fraction prepared from striatal slices cAMP-phosphodiesterase has a biphasic kinetic profile. Incubation of striatal slices with dopamine or opiate receptor agonists changes the biphasic profile to a monophasic function. Haloperidol prevents the changes elicited by both types of agonists. In addition, the changes in PDE kinetic properties and calmodulin content elicited by morphine were also curtailed by naltroxone and striatal deafferentation.

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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. There exists ample evidence for a direct relationship between dopamine receptors and the presence of dopamine-sensitive adenylate cyclase in caudate nucleus. It is well established that the cyclic nucleotide content is regulated by nucleotide cyclases and cyclic nucleotide phosphodiesterases. Both enzymes are regulated by a common Ca^{2+}-dependent regulator protein called calmodulin.

The present experiments were carried out to evaluate the participation of calmodulin in the function of dopamine receptors. It was of interest to establish how the compartmentation of calmodulin and the apparent kinetic properties of PDE can be changed during persistent stimulation of dopamine receptors.

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 phosphate-buffered saline containing Tween 20 (0.05%).

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. Hemitransection of the nigra-striatal fibre bundle was performed 3 weeks before the experiment.

Major Findings: 1. Changes in striatal calmodulin content. 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. Similarly, incubation of striatal slices with morphine (10^{-6}M) increases the calmodulin content in the supernatant fraction and also the amount of calmodulin associated with cAMP-PDE.

2. Changes in the kinetic properties of cAMP-PDE in striatal slices. In control conditions cAMP-PDE in striatal slices exists in at least two kinetic forms, one with a low and one with a high K_m for cAMP. After incubation with dopamine receptor agonists the biphasic double reciprocal plot of initial velocity versus cAMP concentration appears as a monophasic one. Haloperidol (10^{-7}M) prevents this change. Transection of the nigra-striatal fibre bundle by itself does not change the kinetic profile of striatal cAMP-PDE. In slices prepared from deafferented striata activation of dopamine receptors still elicits a decrease in the K_m for cAMP indicating that calmodulin and the calmodulin activatable cAMP-PDE are located in post-synaptic neurons.

Incubation of striatal slices with morphine (10^{-6}M) lowers the K_m for cAMP and changes the kinetic profile frame biphasic to monophasic. These changes in the apparent K_m for cAMP elicited by morphine can be blocked by haloperidol (10^{-7}M) and by naltrexon (10^{-6}M). In slices prepared from deafferented caudate nuclei morphine (10^{-6}M) fails to cause a change in the kinetic properties of cAMP-PDE.

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 activation by glutaminergic or gabaminergic neurons will be studied using calmodulin, adenylate cyclase and cAMP-PDE as biochemical indices.

Publications:

1. Hanbauer, I., Gimble, J. and Lovenberg, W.: Changes in soluble calmodulin following activation of dopamine receptors in rat striatal slices. Neuropharmacology, 1979 (In press).

2. Hanbauer, I., Gimble, J., Sankaran, K. and Sherard, R.: Modulation of striatal cyclic nucleotide phosphodiesterase by calmodulin: Regulation by opiate and dopamine receptors. Neuropharmacology 1979 (In press).

3. Hanbauer, I.: Participation of calmodulin in the regulation of dopamine receptors. In: Catecholamine Basic and Clinical Frontiers (Usdin, E., Kopin, I.J. and Barchas, J., eds.) Pergamon Press, N.Y. Vol. 2, 1979, pps. 1212-1214.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01848-05 HE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Dopamine-β-hydroxylase in Human Cerebrospinal Fluid

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

PI:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI
OTHER:	Pauline Lerner	Research Chemist	BPB NIMH
	Adrian Williams	Clinical Associate	ETB NINCDS
	L. Franklin Major	Clinical Associate	CNB NIMH
	James C. Ballenger	Clinical Associate	BPB NIMH
	Gerald L. Brown	Medical Officer	BPB NIMH

COOPERATING UNITS (if any)
Biological Psychiatry Branch, NIMH; Intramural Research Program, NINCDS

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 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)

Dopamine-β-hydroxylase (DBH) has been measured in cerebrospinal fluid (CSF) from normal volunteers and neurological and psychiatric patients. The DBH levels in CSF from patients with diseases of the basal ganglia were not significantly different from normal volunteers. Among members of a family with dystonia, CSF DBH varied widely, was not related to the severity of the disease, and was not significantly different from normal DBH levels. When alcoholics were treated with disulfiram, there was no change in CSF DBH activity. Basal DBH activity was related to adverse drug reactions. Patients with lower DBH in the CSF were more susceptible to adverse (psychotic and other) reactions to disulfiram. These data are consistent with the known inhibitory effect of disulfiram on DBH in situ. DBH in CSF was also related to personality measures. The patients with low CSF DBH had elevated MMPI profiles, suggesting psychological vulnerability.

5 8 8

Objectives: DBH is found, along with norepinephrine, in storage vesicles of noradrenergic neurons. When noradrenergic nerves fire, both DBH and norepinephrine are released into the synaptic cleft. DBH and norepinephrine from the sympathetic nervous system can be measured in blood. The DBH found in CSF is largely of central origin. We have measured DBH in human CSF as a possible indicator of the integrity or activity of the central noradrenergic system.

Methods: Cerebrospinal fluid is taken from neurological or psychiatric patients or normal volunteers. Because the concentration of DBH in CSF is extremely low, a very sensitive assay is required. We have modified a previously existing radioenzymatic assay method by changing the incubation conditions and by using ^{14}C labeled substrate of very high specific activity. This assay is sensitive enough to reproducibly detect and quantitate the very small amount of DBH present in CSF.

Major Findings: CSF DBH levels were not significantly different between normals and patients with various neurological diseases--Parkinson's, supranuclear palsy, Shy-Drager syndrome, torticollis, and familial dystonia. The subgroup of patients with diseases of the basal ganglia did not significantly differ from each other in their CSF DBH. For the first time, DBH has been measured in six members of a family (mother and five sibs) with hereditary dystonia. The DBH levels in their CSF were within the normal range. The DBH values in these six people covered a 4-fold range and were not related to the severity of the disease. The wide range in the activity of DBH in these family members is interesting in view of the reported relatedness of serum DBH among close relatives.

DBH has also been measured in CSF of alcoholics before and during disulfiram treatment. Disulfiram interferes with ethanol metabolism by inhibiting aldehyde dehydrogenase. Disulfiram also inhibits DBH in situ but diffuses out of CSF, so that no enzyme inhibition was seen in CSF samples. Patients with low basal DBH were particularly susceptible to DBH inhibition in the central nervous system. Each of the 4 patients who had major (psychotic) reactions to the drug had very low DBH in the CSF: those with minor drug reactions had moderate DBH levels; and those with higher DBH had no adverse reactions to the drug. Patients with lower than average DBH in the CSF had elevated MMPI profiles, suggesting that low DBH may be related to psychological vulnerabilities.

Significance to Biomedical Research and Institute Programs: The measurement of DBH activity in CSF appears to be a useful technique for obtaining information on central noradrenergic activity in human subjects. Analysis of DBH in human CSF should help to elucidate the role of the central noradrenergic system in various disease states.

Proposed Course of Project: We plan to compare the DBH activity in CSF with other compounds of neurological interest in CSF and with DBH activity in plasma. We also plan to study DBH in CSF of hypertensives. In addition, we plan to investigate the relationship between CSF DBH and personality measures in normal volunteers.

Publications:

1. Lerner, P., Goodwin, F.K., van Kammen, D.P., Post, R.M., Major, L.F., Ballenger, J.C. and Lovenberg, W.: Dopamine-β-hydroxylase in the cerebrospinal fluid of psychiatric patients. Biol. Psychiat. 13: 685-694, 1978.

2. Major, L.F., Lerner, P., Ballenger, J.C., Brown, G.L., Goodwin, F.K. and Lovenberg, W.: Dopamine-β-hydroxylase in the cerebrospinal fluid: Relationship to disulfiram-induced psychosis. Biol. Psychiatry 14: 337-344, 1979.

3. Lerner, P., Major, L.F., Murphy, D.L., Lipper, S., Lake, C.R. and Lovenberg, W.: Dopamine-β-hydroxylase and norepinephrine in human cerebrospinal fluid: Effects of monoamine oxidase inhibitors. Neuropharmacology 18: 423-426, 1979.

4. Major, L.F., Lerner, P., Goodwin, F.K., Ballenger, J.C., Brown, G.L. and Lovenberg, W.: Dopamine-β-hydroxylase in cerebrospinal fluid: Relationship to personality measures. Arch. Gen. Psychiat., 1979, (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01850-10 HE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Biochemistry of the Spontaneously Hypertensive Rats

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

PI:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI
OTHER:	Y. Yamori	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	1.0	1.0

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

We have reported that the tissue content of taurine is significantly reduced in the stroke-prone spontaneously hypertensive rats and that the inclusion of 3% taurine in the drinking water reduces the severity of hypertension and the incidence of stroke. In separate experiments we have found that the genetically stroke-prone rats have a very low incidence of stroke when they are maintained from weaning on a diet containing 24% protein containing adequate amounts of the sulfur amino acids. It is suggested that an interaction of genetic and environmental factors may be responsible for stroke.

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Objectives: The spontaneously hypertensive rats SHR and the stroke-prone substrain SHR-SP are attractive models for the investigation of biochemical mechanisms in blood pressure regulation and cardiovascular disease. In previous years we have examined neuronal systems related to blood pressure regulation and the hypertrophy of vascular system. This area is under continued investigation. Another area of potential consequence is the role of dietary factors on hypertension and the incidence of vascular disease including stroke. It had been observed that the SHR-SP exhibited a significantly lower incidence of stroke in our laboratory than had been reported in Japan. Since the only obvious difference was in the source of diet a collaborative experiment was begun in which weaning rats from both our colony here and the colony at the Center for Stroke Prevention in Izumo were studied under different dietary regimens.

Methods: Four groups of age-matched male SHR-SP were established at the time of weaning. Two of the groups were derived from the NIH colony and two from the stroke prevention center in Japan. One group from each source received the standard NIH rat chow and the other groups received commercial Japanese rat food. Blood pressure was recorded periodically by a tail cuff method and growth rates of each group recorded. Rats were observed daily and any symptoms of cerebrovascular accidents recorded. The amino acid and total protein content of the diet was determined by amino acid analysis following hydrolysis of diet samples overnight at 110° in 6 N HCl and was performed by automatic analytic techniques.

Major Findings: In prior years we reported that addition of taurine or methionine to standard rat chow result in a small but significant decrease in the blood pressure of the SHR-SP. Furthermore, it appeared that endogenous levels of taurine were decreased in the SHR-SP.

During the first 6 months of the feeding experiment, the four groups of rats grew at similar rates and all became severely hypertensive with each group having mean blood pressures of over 200 mm Hg $^{++}$. By 8 months of age the two groups receiving the Japanese rat chow had a 60% incidence of stroke (either death or neurological symptom). The two matched groups receiving NIH rat chow had no apparent symptoms of stroke. This experiment is still ongoing. It is clear, however, that some dietary component has a major influence on the incidence of cerebrovascular accidents. Examination of the reported nutritional content of the two diets revealed no significant differences, however, amino acid analysis of hydrolysates of the two diets showed that the Japanese diet contained about 17% protein whereas the NIH chow contained 24% protein. Since the exact protein sources are not known it is difficult to compare the quality of the protein. It is of interest, however, that the content of sulfur amine acids was relatively low in the Japanese diet. It is, of course, these sulfure amino acids that appeared to have prophylactic effect on stroke when the ordinary Japanese diets had been supplemented with them (previous work).

Significance to Biomedical Research and Institute Programs: The importance of the interaction between genetic and nutritional factors in human disease is becoming increasingly apparent. Nowhere is this more apparent than in cardiovascular disease. The current experiments show that while the genetic disposition for high blood pressure is clearly one of the most important etiologic factors in stroke, subtle nutritional factors play an equally important role in regulating the incidence of stroke. Given the magnitude of the world population it is clear that increasingly greater emphasis will have to be placed on environmental factors in the prevention of disease, such as hypertension and stroke.

Proposed Course of Project: Additional studies have been initiated to determine the effect of chronic dietary administration of cysteine, methionine and taurine on the development of hypertension in SHR and SHR-SP.

Publications:

1. Nara, Y., Yamori, Y. and Lovenberg, W.: Effect of dietary taurine on blood pressure in genetically hypertensive rats. Biochem. Pharmacol. 27: 2689-2692, 1978.

2. Yamori, Y., Horie, R., Ikeda, K., Nara, Y. and Lovenberg, W.: Prophylactic effect of dietary protein on stroke and its mechanisms. In: Prophylactic Approach to Hypertensive Diseases (Y. Yamori, W. Lovenberg, and E. Freis, eds.) Raven Press, N.Y., 1979 (In press).

3. Lovenberg, W., Nakada, T. and Yamori, Y.: Neuronal regulation of vascular non-collagen protein formation in the spontaneously hypertensive rat. Japaneses Heart Journal, 1979 (In press).

4. Nakada, T., Shigematsu, H. and Lovenberg, W.: Increased protein synthesis in the internal spermatic and testicular arteries of the stroke-prone spontaneously hypertensive rats. Japanese Heart Journal 1979 (In press).

5. Yamori, Y., Nara, Y., Horie, R., Ooshima, A. and Lovenberg, W.: Pathophysiological role of taurine in blood pressure regulation in stroke-prone spontaneously hyertensive rats (SHR). In: The Action of Taurine on Excitable Tissue, (S. Schaffer and S. Baskin, eds.) Spectrum Press, N.Y., 1979 (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01851-05 HE

PERIOD COVERED
October 1, 1978 to September 30, 1979

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	NIH Postdoctorate Fellow	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The primary goal of these studies is an understanding of the regulation of tyrosine hydroxylase in the central nervous system. We have previously shown an activation of the soluble enzyme by phosphorylation or catecholamine removal. Tyrosine hydroxylase activity is now being studied in a synaptosomal system, which provides conditions more similar to those found in vivo. Synaptosomes prepared from mouse whole brain and rat striatum have been used to study the regulation of tyrosine hydroxylase. Removal of calcium and chronic morphine treatment decreases tyrosine hydroxylase activity in synaptosomes from mouse whole brain but not in those from striatum. Conversely, addition of the calcium ionophore A23187, results in increased tyrosine hydroxylase activity in synaptosomes from both whole brain and striatum. We have also found that tyrosine hydroxylase activity in whole brain synaptosomal preparations is dependent on extrasynaptosomal tyrosine concentration, and that removal of calcium changes the kinetics of tyrosine hydroxylase for tyrosine. Tyrosine hydroxylase activity in striatal synaptosomes did not show this tyrosine dependency.

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Objectives: As the rate-limiting enzyme in catecholamine biosynthesis in the central nervous system, tyrosine hydroxylase is intimately involved in the regulation of neurons utilizing catecholamine neurotransmitters. In previous reports we have described some aspects of regulation of tyrosine hydroxylase activity in soluble enzyme preparations. The objective of our continued studies is to better describe the mechanisms involved in regulation of tyrosine hydroxylase activity under conditions more similar to those found in vivo. This preparation allows us to study several of the factors involved in regulation of the enzyme, including concentration of substrate and cofactor, end-product inhibition, ion effects; and their interactions. Since many drugs which have been shown to affect norepinephrine and dopamine turnover in vivo have not been shown to affect tyrosine hydroxylase activity using a soluble enzyme assay, we will investigate the effects of drugs administered in vivo on tyrosine hydroxylase activity in synaptosomal preparations.

Methods: Synaptosomes were prepared from mouse whole brain and rat striatum using standard procedures. The crude synaptosomal pellet or synaptosomes purified on a sucrose density gradient were resuspended in a modified Krebs buffer without calcium and containing 0.32 M. sucrose. Tyrosine hydroxylase activity was quantitated by measuring formation of tritiated water after addition of ^{3}H-tyrosine. For the standard assay each tube contained 150-250 µg protein, 5 x 10^{-5}M unlabelled tyrosine and Ca^{2+}-free or Ca^{2+}-buffer (final Ca^{2+} concentration = 1 mM). Tyrosine concentrations were varied from 5 x 10^{-6}M to 1 x 10^{-4}M. Ca^{2+}-ionophores were added in either Ca^{2+}-free or Ca^{2+}-containing buffers. Tubes were incubated at 37°C for 10 min. The reaction was stopped with 0.4 ml 5% TCA. Samples were put over columns containing Dowex 50 x 4, activated charcoal and Dowex 1 x 2; the columns were washed twice with 0.7 ml H_2O. The effluent was collected in scintillation vials and $^{3}H_2O$ was quantitated by liquid scintillation spectrometry. Animals were treated both acutely and chronically with morphine. Acute treatment consisted of 30 mg/kg s.c. for rats and 100 mg/kg s.c. for mice. Thirty minutes after injection the animals were decapitated. Mice and rats were rendered tolerant to and dependent on morphine by implantation of a pellet containing 75 mg morphine base. Animals were decapitated 72 hr after pellet implantation.

Major Findings: Tyrosine uptake into the synaptosomal preparations was rapid and essentially complete after two minutes exposure to various tyrosine concentrations (5 x 10^{-6}, 1 x 10^{-5}, 2 x 10^{-5}, 5 x 10^{-5} and 1 x 10^{-4}M). Tyrosine uptake was essentially linear over this concentration range.

In synaptosomes obtained from mouse whole brain and rat frontal cortex the rate of tyrosine hydroxylation, expressed as pmole DOPA/mg protein/min, is dependent on the concentration of tyrosine in the external media. The rate of formation of DOPA is linear up to 50 µM and begins to be saturated at 100 µM. Conversely, in synaptosomes obtained from rat striatum the rate of tyrosine hydroxylation is not dependent on the concentration of external tyrosine, the relationship between tyrosine hydroxylation and tyrosine concentration being flat over the range of tyrosine concentrations tested.

The rate of tyrosine hydroxylation can be affected by manipulating extra- and intrasynaptosomal Ca^{2+} concentrations. These effects may vary depending on the brain region studied. Tyrosine hydroxylation in synaptomes obtained from whole brain was decreased by approximately 30% when incubated in Ca^{2+}-free buffer. Incubation without Ca^{2+} also changed the characteristics of the relationship between tyrosine concentration and tyrosine hydroxylase activity in whole brain synaptosomes. The rate of tyrosine hydroxylation in Ca^{2+}-free buffer was linear from 10-100 µM tyrosine and was lower at each concentration of tyrosine when compared to synaptosomes incubated in the presence of Ca^{2+}. The rate of tyrosine hydroxylation in striatal synaptosomes was unaffected by removal by Ca^{2+} from the incubation medium.

A Ca^{2+}-ionophore, A23187, was used to increase intrasynaptosomal Ca^{2+} concentrations, as shown by increased $^{45}Ca^{2+}$ uptake into synaptosomal preparations. A23187 increased the rate of tyrosine hydroxylation to approximately 160% of control in both whole brain and striatal synaptosomes in the presence of 5×10^{-5}M tyrosine. This effect was not seen when A23187 was added in the absence of Ca^{2+}. A23187 had no effect on the activity of soluble tyrosine hydroxylase. Under the incubation conditions used A23187 decreased tyrosine uptake by approximately 25% and caused a 60% increase in dopamine release from striatal synaptosomes, the latter effect was not reversed by removal of extracellular Ca^{2+}. The effect of A23187 on the rate of tyrosine hydroxylation in striatal synaptosomes increased with increasing extrasynaptosomal tyrosine concentration. This effect is most likely not due to higher concentrations of tyrosine overcoming the decreased tyrosine uptake caused by the ionophore since the control enzyme activity is not dependent on the tyrosine concentration.

Morphine, which may exert some of its effects by affecting Ca^{2+} availability, had different effects on synaptosomal tyrosine hydroxylase activity, depending on the type of synaptosomes and type of treatment. Acute morphine treatment had no effect on the rate of tyrosine hydroxylase in synaptosomes from mouse whole brain or rat striatum.

When synaptosomes were incubated in buffer containing Ca^{2+}, tyrosine hydroxylation was significantly reduced in synaptosomes obtained from mice chronically-treated with morphine as compared to placebo implanted controls. However, when synaptosomes were incubated in Ca^{2+}-free buffer, this difference was no longer apparent, indicating some interaction between Ca^{2+} and morphine on tyrosine hydroxylase activity. In contrast, the tyrosine hydroxylase activity in striatal synaptosomes prepared from rats chronically-treated with morphine was unchanged. Based on studies with a soluble tyrosine hydroxylase system several factors, such as tyrosine concentration, were considered to contribute little to the regulation of tyrosine hydroxylase activity. The results of these studies indicate that tyrosine and Ca^{2+} concentrations may contribute significantly to the regulation of this enzyme in vivo. We have also demonstrated that the characteristics of regulation may differ depending on the area of the brain studied. In addition, we have shown an effect of chronic morphine treatment on tyrosine hydroxylase activity, a drug which has been shown to affect catecholamine turnover but had not been demonstrated to have an effect on the rate-limiting enzyme.

Significance to Biomedical Research and Institute Program: The regulation of tyrosine hydroxylase activity has been studied in soluble enzyme preparations in an attempt to understand the regulation of this enzyme in vivo. Several important control mechanisms have been defined, such as phosphorylation, cofactor concentration and catecholamine end-product inhibition. However, the soluble enzyme preparation seems to lack some sensitivity in that the enzyme is removed from conditions normally found in vivo, as evidenced by the difficulty in observing effects of drugs, known to effect catecholamine turnover or synthesis, on tyrosine hydroxylase activity. The synaptosomal preparation used in these studies provides us with a means of studying the regulation of tyrosine hydroxylase under conditions similar to those found in vivo. Such a system can provide important information concerning the interaction of drugs with central catecholaminergic neural systems.

The regulation of tyrosine hydroxylase may be important in disease states with central nervous system involvement. For example, increasing brain tyrosine concentrations has been shown to lower blood pressure in spontaneously hypertensive rats. The work reported here may provide an explanation for this phenomenon.

Proposed Course of Project: Since differences were found between whole brain and striatum, the regulation of tyrosine hydroxylase activity in synaptosomal preparations from hypothalamus and pons medulla, as well as striatum and cortex will be studied. Further characterization will include further study of tyrosine and ion interactions with tyrosine hydroxylase activity as well as cofactor concentrations, end-product inhibition and phosphorylation of synaptosomal proteins.

Characterization of tyrosine hydroxylase in the central nervous system would enhance the understanding of the role of regulation of this enzyme in the central nervous system. Therefore, further work will be done on the purification of tyrosine hydroxylase. Studies on purified tyrosine hydroxylase from difference brain regions will provide a different aspect on the regulation of tyrosine hydroxylase in the central nervous system.

Publications:

Young, R.A., Robinson, D.S., Vagenakis, A.G., Saavedra, J.M., Lovenberg, W., Krupp, P.P. and Danforth, E.,Jr.: Brain TRH, monoamines, tyrosine hydroxylase, and tryptophan hydroxylase in the woodchuck, Marmota monax, during the hiberation season. Comparative Biochem. and Physiol. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01859-08 HE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Characterization and Mechanism of Action of Dopamine-β-Hydroxylase

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

PI:	Sitka Pradhan	Staff Fellow	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

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 soluble and membrane-bound forms of dopamine-β-hydroxylase (DBH) are being isolated from bovine adrenal medulla and purified. The enzyme will then be treated with glycosidases to remove the carbohydrate moieties from the DBH molecules. The biologic and immunologic properties of DBH will be checked both before and after this cleavage. A sequence study will also be performed on the carbohydrate components of DBH molecules.

598

PHS-6040
(Rev. 10-76)

Objectives: The main objective of the project is to study the carbohydrate components of dopamine-β-hydroxylase (both soluble and membrane-bound forms).

Methods: The soluble and membrane-bound forms of dopamine-β-hydroxylase (DBH) have been isolated from bovine adrenal medulla according to Foldes et al. [Biochem. J. (1972) 126: 1209-1219]. Both forms of the enzymes have been purified by passing them through a column of DEAE cellulose. Concanavalin A Sepharose and Sephacryl S-300 successively. The homogeneity of the final purified preparation is examined by SDS-gel electrophoresis. The purified DBH will then be treated with glycosidase enzymes prepared from Diplococcus pneumoniae, Type 1, to cleave the carbohydrate moiety from DBH molecule.

Major Findings: Our preliminary results show that the carbohydrate moiety can be cleaved without any loss of biological activity of DBH.

Significance to Biomedical Research and Institute Programs: DBH is the final enzyme in the catalytic pathway for the synthesis of norepinephrine. This enzyme is located in the synaptic and chromaffin granule vesicles. Upon nerve stimulation it is released with the neurotransmitter by an exocytotic process. Hyperactivity of the sympathetic nervous system has been implicated in the development and maintenance of essential hypertension in man and experimental models of hypertension in animals. A clear knowledge of the molecular structure of DBH is required for a complete understanding of its role in both the normal and pathologic state.

Proposed Course of Project: We are currently pursuing the isolation and purification of DBH in large quantities to determine if any biologic or immunologic activity is associated with the carbohydrate moiety. We also plan to sequence this carbohydrate component of the DBH molecule.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01865-04 HE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Calcium Dependent Activation of Tryptophan Hydroxylase by ATP and Magnesium

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

PI:	Donald Kuhn	Staff Fellow	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem.Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	

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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)

Tryptophan hydroxylase [EC 1.14.16.4; L-tryptophan, tetrahydropteridine: oxygen oxidoreductase (5-hydroxylating)] in rat brainstem extracts is activated 2 to 2.5-fold by ATP and Mg^{++} in the presence of subsaturating concentrations of the cofactor, 6-methyltetrahydropterin ($6MPH_4$). The activation of tryptophan hydroxylase under these conditions results from a reduction in the apparent K_m for $6MPH_4$ from 0.21 mM to 0.09 mM. The activation requires Mg^{++} and ATP but is not dependent on either cAMP or cGMP. The effect of ATP and Mg^{++} on enzyme activity was enhanced by μM concentrations of Ca^{++} and totally blocked by EGTA. These data suggest that tryptophan hydroxylase can be activated by a cyclic nucleotide independent protein kinase which requires low calcium concentrations for the expression of its activity.

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Objectives: It has been demonstrated that tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of the catecholamines, dopamine and norepinephrine, can exist in two different activity states corresponding to forms having a high and a low Michaelis constant for the reduced pterin cofactor. Furthermore, the interconversion of tyrosine hydroxylase from one form to the other is apparently mediated by a cAMP dependent protein kinase (Lovenberg et al., PNAS 72:2955-2958, 1975). In an effort to learn more about the control of tryptophan hydroxylase (TPH), studies similar to those with tyrosine hydroxylase were undertaken to determine if protein phosphorylation might also modulate the activity of this enzyme.

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), cAMP (0.2 mM), and Mg^{++} acetate (10 mM).

Major Findings: Exposure of tryptophan hydroxylase to phosphorylating components leads to a 2 to 2.5-fold stimulation of catalytic activity. This effect is rapidly reversible. ATP, cAMP, and Mg^{++} reduce the apparent K_m for $6MPH_4$ from 0.21 mM to 0.09 mM while having no effect on V_{max}. Kinetics for the substrate tryptophan were not changed. The stimulation of enzyme activity was dependent on ATP and Mg^{++}, but not upon cAMP. The activation by ATP-Mg^{++} was still independent of cAMP after removal of endogenous cAMP by gel filtration of the enzyme on Sephadex G-25. Neither cGMP nor dibutyryl cAMP had any further effect on the ATP-Mg^{++} activation. The ATP analog AMP-P(NH)-P could not replace ATP in the activation reaction. The addition of cAMP-dependent protein kinase was also without further stimulation. The cAMP-dependent protein kinase inhibitors adenosine, adenine, ADP, and the Walsh inhibitor did not block the ATP-Mg^{++} activation. The inclusion of NaF in the reaction mixture, which partially inhibits phosphatases, slightly enhanced the phosphorylation effect.

The ATP-Mg^{++} effect on tryptophan hydroxylase was seen in all brain areas tested including the mesencephalic tegmentum, tectum, hypothalamus, septum, and pineal gland. The effect was also apparent in the tegmentum when subsaturating concentrations of either tetrahydrobiopterin or dimethyltetrahydropterin were used in place of $6MPH_4$.

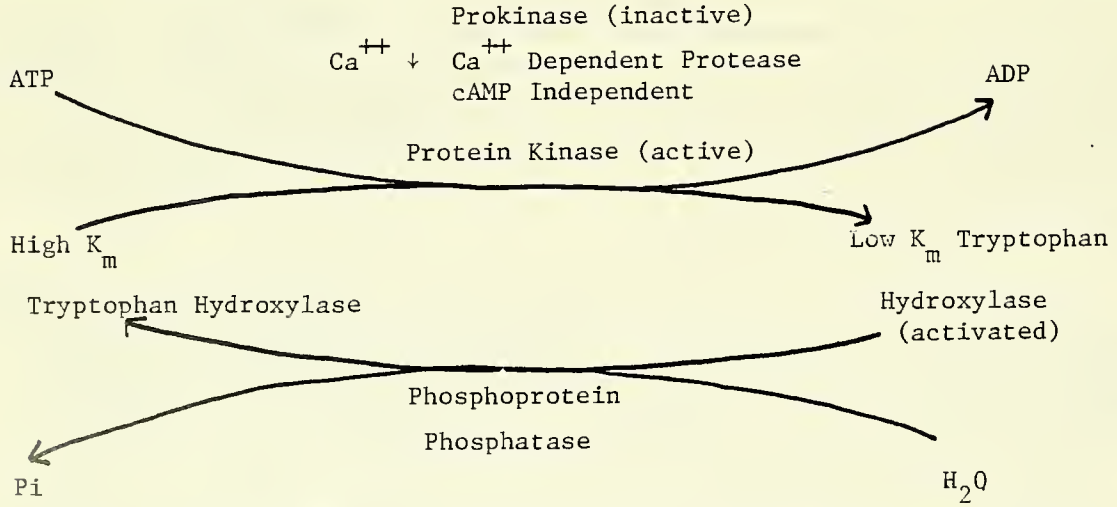
The divalent cation calcium was also found to play an important role in the activation of tryptophan hydroxylase. Concentrations of Ca^{++} as low as 5-10 μM stimulate the ATP-Mg^{++} effect. Homogenization of brain tissue in the presence of EGTA prevented the activation of tryptophan hydroxylase by ATP-Mg^{++} which, in turn, can be reinstated by the addition of Ca^{++} in excess of EGTA. The addition of EGTA to the reaction mixtures also blocks the ATP-Mg^{++} activation.

601

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^{++} was found to play an important role in the activation. Preliminary experiments indicate that Ca^{++} is necessary for the conversion of an inactive protein kinase proenzyme to an active kinase (by a protease) which, then, can phosphorylate tryptophan hydroxylase. In addition, significant cAMP independent protein kinase activity was measured in the tryptophan hydroxylase containing extracts.

Proposed Course of Project: The following studies are planned to more completely assess the effects of phosphorylation on tryptophan hydroxylase. The prokinase and protease enzymes described will be purified as will tryptophan hydroxylase. With purified enzymes the following reaction scheme for the activation of tryptophan hydroxylase will be tested.



The role of calmodulin in the Ca^{++}-dependent ATP-Mg^{++} stimulation of tryptophan hydroxylase will also be more extensively investigated. Finally, we will try to answer the question of whether or not the enzyme itself is directly phosphorylated.

Publications:

1. Lovenberg, W. and Kuhn, D.M.: Role of hydroxylase cofactor in serotonin synthesis. Psychopharmacology Bulletin 14: 44-46, 1978.

2. Kuhn, D.M., Vogel, R., and Lovenberg, W.: Calcium-dependent activation of tryptophan hydroxylase by ATP-magnesium. Biochem. Biophys. Res. Comm., 82: 759-766, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01867-04 HE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Neuroleptic Regulation of Tyrosine Hydroxylase
(Revised Title)

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

PI:	Larry Alphs	Research Associate	HE NHLBI
OTHER:	Sitka Pradhan	Staff Fellow	HE NHLBI
	Eleanor Bruckwick	Research Assistant	HE NHLBI
	Walter Lovenberg	Chief, Sect. Biochem.Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.7	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 goal of this work is to characterize changes in tyrosine hydroxylase activity in the rat striatum after acute and subacute administration of haloperidol. To determine the optimum conditions under which changes in tyrosine hydroxylase activity are observed we have examined in detail the effects of pH and buffer systems on enzyme activity. We have also attempted to correlate changes in tyrosine hydroxylase activity in animals treated with neuroleptics with changes in adenylate cyclase and cAMP-dependent kinase.

604

Objectives: The objective of this project is to study the mechanisms whereby neuroleptics regulate tyrosine hydroxylase in the rat striatum. To do this we are seeking to determine the optimum conditions under which neuroleptic-induced changes in tyrosine hydroxylase are observed. We are also seeking to correlate changes in tyrosine hydroxylase with changes in adenylate cyclase and cyclic AMP-dependent kinase.

Methods: A standard tritium release assay is used to measure changes in tyrosine hydroxylase activity. Steady state kinetic techniques are also employed to examine changes in the kinetic properties of the enzyme after pretreatment of animals with neuroleptics. Adenylate cyclase is measured by a standard enzymatic method in which cAMP is generated and subsequently assayed by radioimmunoassay. Cyclic AMP-dependent kinase is measured by a standard radioisotopic method.

Major Findings: Kinetic properties of tyrosine hydroxylase have been demonstrated to be markedly affected by pH. As the pH of the reaction mixture is made more alkaline there is a marked increase in K_m. Incubating striatal homogenates in the presence of phosphorylating conditions increases tyrosine hydroxylase activity and shifts the pH optimum to the right. When rats are treated subacutely for 10 days with haloperidol (1.0 mg/kg) and the kinetic parameters of tyrosine hydroxylase activity are examined at 1 hour and 23 hours after the last injection, the K_m is decreased from control values at 1 hour and rises above control values at 23 hours. In acutely treated animals there is a decrease from control in K_m at 1 hour after injection which returns to control levels by 23 hours. The K_i for the inhibition of striatal tyrosine hydroxylase by dopamine is subacutely treated rats is elevated about 2-fold.

Dopamine stimulable adenylate cyclase activity increases above control values 1 hour after a single injection of haloperidol (5.0 mg/kg), while a similar dose in animals having received 10 daily injections of haloperidol results in a decrease from control in adenylate cyclase activity at 1 hour. Baseline levels of adenylate cyclase activity are also suppressed in these subacutely treated animals.

Similarly, cAMP-dependent kinase in the rat striatum is increased above controls 1 hour after acute treatment with haloperidol (5.0 mg/kg) and is decreased 1 hour after the last of 10 daily injections of the neuroleptic.

Significance to Biomedical Research and Institute Programs: Since tyrosine hydroxylase is the rate-limiting enzyme in the biosynthesis of catecholamine neurotransmitters, 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. Among the pathologic states that might potentially be affected by these studies are conditions as diverse as essential hypertension, Parkinson's disease, Huntington's chorea and schizophrenia. Since current evidence implicates other enzymes, including cAMP-dependent kinase and adenylate cyclase, in the short-term regulation of tyrosine hydroxylase activity, it is also of interest to observe the effects of neuroleptics on these enzymes.

Proposed Course of Project: Future work will be directed at trying to further identify those parameters which allow for optimal characterization of the effects of neuroleptics. Specifically, time course and dose effect studies of haloperidol are planned. Attempts will be made to reduce the effects of phosphatase in our assay system by homogenizing rat striata in the presence of sodium fluoride. We also plan studies to determine the effects of neuroleptics in other areas of the central nervous system as well as on the ontogeny of tyrosine hydroxylase, adenylate cyclase and cAMP-dependent kinase.

Publications:

Lovenberg, W., Alphs, L., Bose-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., 1979 (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01868-04 HE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Regulation of Tyrosine Hydroxylase

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

PI:	Judith Juskevich	NIH Postdoctoral Fellow	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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)

Clonidine is a centrally-acting α-agonist used clinically to control hypertension. We have shown that clonidine decreases MOPEG-SO_4, a metabolite of norepinephrine, levels in the brain. This indicates that clonidine decreases turnover or synthesis of norepinephrine which should be reflected in changes in tyrosine hydroxylase activity. We previously found that acute or chronic treatment of spontaneously hypertensive rats (SHR) with clonidine does not affect the apparent K_m of tyrosine hydroxylase for the synthetic cofactor, 6-methyltetrahydropterine, when measured in vitro in several different brain regions. Wistar-Kyoto (WKY) rats were acutely treated with clonidine and tyrosine hydroxylase activity was measured in forebrain, hippocampus, hypothalamus, pons-medulla and striatum. Clonidine caused a small, but significant increase in the K_m and V_{max} for cofactor in the forebrain. Tyrosine hydroxylase activity was unaffected in the other brain regions. Clonidine withdrawal after ten days of treatment caused a sustained increase in the V_{max} of tyrosine hyroxylase from the pons-medulla.

607

PHS-6040
(Rev. 10-76)

Objectives: Central nervous system catecholaminergic pathways are possibly involved in the pathogenesis or maintenance of hypertension in the spontaneously hypertensive rat. Since tyrosine hydroxylase is the rate-limiting enzyme in the biosynthesis of catecholamine neurotransmitters, we studied tyrosine hydroxylase activity in different brain regions of SHR and WKY rats. Clonidine is a centrally-acting antihypertensive agent which lowers blood pressure by affecting central catecholaminergic pathways. We, therefore, studied the effects of clonidine on tyrosine hydroxylase activity in different areas of the brains of SHR and WKY rats treated with clonidine. Withdrawal of clonidine has been reported to cause an overshoot of blood pressure in hypertensive patients. Therefore, we studied the effects of clonidine withdrawal on tyrosine hydroxylase activity in the brains and adrenals of SHR's treated with clonidine for ten days to determine whether rebound changes in enzyme activity could be responsible for the overshoot in blood pressure seen in hypertensive patients.

Methods: Male WKY and SHR rats, 16 weeks of age, were used for all experiments. Acute treatment with clonidine consisted of a single injection of 300 µg/kg administered intraperitoneally. Control animals were injected with saline. Animals were decapitated 3 hours after injection and brain regions were dissected according to standard procedures. For chronic administration of the drug, clonidine was dissolved in the drinking water such that each animal received approximately 300 µg/kg/day. Both treatments have been shown to decrease blood pressure in SHR.

To study the effects of clonidine withdrawal animals were sacrificed 12,24,48,72 and 96 hours after clonidine was replaced with tap water.

Major Findings: In previous experiments we found that acute and 5 or 10 day treatments of SHR with clonidine had no effect on tyrosine hydroxylase activity in any brain region studied although acute treatment decreased MOPEG-SO_4 levels. We also found no difference in tyrosine hydroxylase activity in adrenals from treated and non-treated SHR.

There was a small but significant increase in the K_m and V_{max} of tyrosine hydroxylase from the forebrain of WKY rats treated acutely with clonidine. No differences were found in hippocampus, hypothalamus, pons-medulla or striatum of acutely treated WKY's.

Tyrosine hydroxylase activity was measured in adrenals, pons-medulla, hypothalamus and forebrain from animals treated with clonidine for ten days and withdrawn from drug treatment. In adrenals the K_m of tyrosine hydroxylase for cofactor remained unchanged 12,24,48,72 and 96 hours after drug removal. The V_{max}, however, increased 24 hours after removal of clonidine and remained elevated at 96 hours (the last time point tested).

Although incomplete, the data from the different brain regions show that the increase in tyrosine hydroxylase activity after withdrawal of clonidine is not a widespread phenomenon. In pons-medulla the V_{max} of tyrosine hydroxylase increased at 12 hours and remained elevated at 96 hours. There was also a slight increase in the K_m for cofactor at all time points. There were no

changes in tyrosine hydroxylase activity in hypothalamus or forebrain.

Significance to Biomedical Research and Institute Programs: The precise mechanism by which centrally-acting antihypertensive agents exert their effect is not known. It is possible that changes in central or peripheral tyrosine hydroxylase activity contribute to the chronic effects of clonidine and the rebound increase in sympathetic activity seen after withdrawal of the drug.

Since central nervous system differences may be important in the pathogenesis of hypertension in the SHR, clonidine can be used to determine if there are differences in catecholaminergic systems of WKY's and SHR's.

Proposed Course of Project: The effect of chronic clonidine treatment on tyrosine hydroxylase activity in different brain regions of WKY rats will be completed, in order to determine whether SHR and WKY rats respond similarly to clonidine treatment.

The changes in MOPEG-SO_4 levels produced by clonidine administration reflect an effect of this drug on central catecholaminergic function. Since tolerance develops to the blood pressure lowering effects of clonidine and rebound hypertension has been noted after withdrawal we will extend this study to examine the effects of 10-day treatments and the withdrawal of clonidine on MOPEG-SO_4 levels in different areas of the rat brain.

The study of effects of clonidine withdrawal will be extended to include hippocampus and striatum.

Since drug treatments may cause a shift in the pH maximum of tyrosine hydroxylase, it is possible that differences in enzyme activity after clonidine treatment may be masked by measuring activity at a single pH. We therefore intend to look into the possibility that clonidine causes a shift in the pH maximum of tyrosine hydroxylase.

We also intend to study the regulation of tyrosine hydroxylase and effects of clonidine in synaptosomal preparations from SHR and WKY rats. Since this preparation is more similar to in vivo conditions, it may be possible to detect differences in enzyme activity in WKY and SHR rats.

Publications:

Lerner, P., Nose, P., Ames, M.M. and Lovenberg, W.: A modification of the tyrosine hydroxylase assay: Increased enzyme activity in the presence of ascorbic acid. Neurochem. Res. 3: 641-651, 1978.

Robinson, D.S., Campbell, I.C., Walker, M., Lovenberg, W., Statham, N.J., and Murphy, D.L.: Effects of chronic monoamine oxidase inhibitor treatment on biogenic amine metabolism in rat brain. Neuropharmacol. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01872-03 HE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Pteridine Cofactor Levels in Biologic Fluids

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

PI:	Robert Levine	Chemist	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI
	Donald Calne	Chief, Exp. Ther. Branch	ETB NINCDS
	Adrian Williams	Clinical Associate	ETB NINCDS

COOPERATING UNITS (if any)
NIMH, Biological Psychiatry Branch; NINCDS, Experimental Therapeutics Branch

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.5	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 investigation of the major determinants of monoamine synthesis and turnover in vivo is of intense scientific interest because monoamine levels in the central nervous system (CNS) play critical roles in neuropsychiatric, neuroendocrine and cardiovascular diseases. Tyrosine hydroxylase, which requires tetrahydrobiopterin (BH_4) as a cofactor, is known to be the rate-limiting enzymatic step in the synthesis of dopamine and norepinephrine. Current evidence suggests that in vivo rate of synthesis depends primarily on dopamine concentration (due to end-product inhibition) and hydroxylase cofactor (BH_4) levels. Thus, cofactor levels in cerebrospinal fluid (CSF) are of interest.

This study was undertaken to detect and monitor CSF hydroxylase cofactor activity using a phenylalanine hydroxylase assay. Our studies indicate hydroxylase cofactor activity declines with age in both normal and diseased patients. No significant alteration was found in CSF cofactor levels of schizophrenic or affective disorder patients. We have reported that patients with certain neurological diseases, most notably Parkinson's disease, have reduced levels of cofactor in their CSF.

610

Objectives: Since a variety of diseases may result from defects in monoamine synthesis, it is of interest to assess one possible index of in vivo aminergic function, i.e., CSF hydroxylase cofactor activity. Studies of neurologic and psychiatric patients are in progress in collaboration with the Biological Psychiatry Branch, NIMH and the Neurology Service (Dr. Calne). In addition, we are investigating cofactor levels in human CSF using an independent method of assay, a high-pressure liquid chromatographic system. Association between CSF cofactor activity and disease states, drug treatments and biologic variables such as age, sex etc. will be investigated.

Methods: Two ml of CSF is freshly collected in an opaque polycarbonate tube, wrapped in aluminum foil to protect it from light and stored in liquid N_2 until time of assay.

After thawing 500 µl of a cerebrospinal fluid sample (0.5 ml) is transferred to each of three 1.5 ml conical polypropylene tubes and lyophilized to dryness immediately prior to assaying. Assay reaction mixture contains 13.0 units sheep liver quinoid dihydropterin reductase (QDPR), 0.85 µmol NADH, 36 units of highly purified phenylalanine hydroxylase, 10 µmol KPO_4 pH 6.8, 400 units catalase, and .01 µmol 4-^{3}H-L-phenylalanine (30 µC/µmol) in a final volume of 70 µl per tube. Following incubation for 45 min. at 30°, the reaction mixture is cooled to 0° in an ice bath, and the reaction arrested by addition of 50 µl of 1.2 M sodium acetate buffer, pH 5.5. To release any tritium in the 3-or 5-position of the tyrosine formed, 25 µl of N-iodosuccinamide (50 mg/ml in DMSO) is added to the cooled samples. After 5 min the released tritium (as ^{3}HOH) is collected by passage of the reaction mixture over a 0.6 x 3 cm Dowex 50-H + exchange resin. The column is washed twice with 0.7 ml of water, and the total eluate collected in a scintillation vial to which 15 ml of scintillation cocktail is added. A calibration curve using tetrahydrobiopterin (BH_4) standards in bovine serum albumin (0.4 mg/ml) are run concomitantly. A UV spectrum is obtained on a 100 µM solution of BH_4 in 0.01 N HCl just prior to the assay to quantify the concentration of reduced cofactor in the cofactor stock solution. CSF cofactor activity is expressed as p-moles per ml CSF.

Major Findings: We have been able to measure hydroxylase cofactor content in all CSF samples assayed from patients with various neurological diseases, schizophrenia, affective disorders, and normal control patients. Preliminary analysis of data collected from schizophrenic and affective disorder patients indicates no detectable alterations in CSF cofactor content when compared to normal controls. However, a larger sample population will be analyzed before these findings are determined conclusively. Results from the neurological and normal patients populations proved to be most interesting. Within the normal patient population, CSF cofactor content decreases with age. In addition, it appears that there is an increasing gradient in cofactor concentration with increasing height (towards the cerebral ventricles) in the spinal column. For these reasons, it was important for all the CSF samples assayed to be obtained from the same milliliter aliquot and our published data compares values of CSF cofactor between diseased patients and controls who were age-matched. Ten patients with untreated Parkinson's disease exhibited approximately 50% lower

cofactor content (8.93 pmol/ml) than normal controls (17.72 pmol/ml). Patients with Progressive Supranuclear Palsy and the Shy-Drager syndrome also exhibited significantly reduced levels. In general, CSF cofactor levels correlate well with CSF HVA content in the combined normal and parkinsonian population ($r = 0.76$, $p < .001$). These results indicate that CSF cofactor levels could serve as a good index of central aminergic activity.

Significance to Biomedical Research and Institute Programs: (1) It is important to identify the major determinants of monoamine synthesis and turnover in man, and to assess the relationships of these variables to biologic variables and disease states. (2) Measurement of CSF cofactor activities in patients have revealed cofactor deficiencies in certain neurological disease states where impaired monoamine metabolism has been shown to be an etiologic factor (such as basal ganglia degeneration). (3) This project will provide additional insight into mechanisms which control CNS monoamine levels and their relationships to pathologic conditions. (4) Our findings of reduced cofactor levels in parkinsonian patients may provide the basis for successful replacement therapy with exogenous BH_4.

Proposed Course of Project: Assay of patient CSF speciments will be continued in order to enlarge our series of patients with neurologic and psychiatric diseases. In addition, we hope to obtain CSF specimens from patients with other illnesses, including cardiovascular disorders, hypertension etc., and from normal controls. Preliminary experiments indicate patients with dystonia (a neurological condition) have very low cofactor levels. We will continue our investigations into the relationship between reducted cofactor content and the condition of dystonia.

We also intend to develop more sensitive and specific methods to measure cofactor levels in biologic fluids, specifically, a high pressure liquid chromatographic system. We hope to expand this project to examine control mechanisms of BH_4 biosynthesis in various tissues.

Publications:

1. Robinson, D.S., Levine, R., Statham, N. and Williams, A.: Hydroxylase cofactor in human CSF - An index of central aminergic activity. Psychopharmacology Bull. 14: 49-51, 1978.

2. Lovenberg, W., Levine, R.A., Robinson, D.S., Ebert, M., Williams, A.C. and Calne, D.B.: Hydroxylase cofactor activity in cerebrospinal fluid of normal subjects and patients with Parkinson's Disease. Science 204: 624-626, 1979.

3. Levine, R.A., Williams, A.C., Robinson, D.S., Calne, D.B. and Lovenberg, W.: Analysis of hydroxylase cofactor activity in human cerebrospinal fluid of patients with Parkinson's Disease. In: Advances in Neurology, Raven Press, N.Y. , 1979 (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01874-03 HE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

The Role of Epinephrine and Epinephrine Containing Neurons in Brain and Adrenal Gland.

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

PI:	Willa B. Phyall	Research Associate	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 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 purpose of this project is to determine whether epinephrine and its biosynthetic enzyme (phenylethanolamine N-methyltransferase) in the central nervous system play a major role in the development of the hypertension in the spontaneously hypertensive rat. While the role of epinephrine and PNMT in the adrenal gland has been studied quite extensively, such studies have not been extended to the brain. Results from our laboratory as well as the literature suggests that (1) there is not a direct relationship between central epinephrine levels, PNMT activity and blood pressure. Inhibitors of PNMT have been found to be hypotensive agents, although evidence suggests that blood pressure reduction is not necessarily related to enzyme inhibition. Reduction of blood pressure by other hypotensive agents does not alter PNMT activity in brain.

PHS-6040
(Rev. 10-76)

Objectives: One of the major objectives of this work is to determine whether adrenergic neurons are involved in blood pressure regulation. We, therefore, undertook experiments to evaluate the biochemical and regulatory properties of phenylethanolamine N-methyltransferase (PNMT) in the central nervous system. Secondly, we attempted to determine if there is any relationship between the activity of this system in the brainstem and the development of hypertension in the spontaneously hypertensive rats. Finally, we wished to determine whether inhibition of PNMT would have any effect on the development of hypertension.

Methods: Male normotensive and spontaneously hypertensive rats (SHR) are used in these studies. To determine the effect of age, SHR in addition to their age-matched Wistar-Kyoto (WKY) controls are sacrificed at 4 and 16 weeks of age. Hypertensive animals used in drug studies are sacrificed after a specified period of drug treatment. Prior to killing, blood pressure is recorded either by the indirect (tail cuff) method or direct method (arterial cannulation). Immediately following sacrifice of the animals, the adrenal glands and brain tissues are removed and frozen on dry ice. The C_1 and C_2 regions are dissected from the medulla oblongata and all tissues are stored in liquid nitrogen until assay. Male rats are used in all spinal cord experiments. Animals are sacrificed and the spinal cords removed intact. The tissue is immediately frozen on dry ice. The cord is then dissected into cervical, thoracic and lumbar regions. Each region is in turn dissected into its respected dorsal and ventral horns. The thoracic region, in addition to the dorsal and ventral horns, the zona intermedia is also dissected. PNMT is measured by a modification of the method employed by Saavedra et al., Nature 248: 1974, while epinephrine levels are determined by the gas chromatographic-mass spectrometric procedure.

Major Findings: The major findings to date show that (1) there is a significant increase in brain PNMT (both C_1 and C_2 regions) in young (4 wk old) SHR rats in comparison to their age-matched WKY-controls. Systolic blood pressure is also significantly higher in the SHR. While both blood pressure and brain enzyme activity are elevated in the hypertensive rats, there is no corresponding elevation in brain epinephrine content. (2) In the adult SHR rats, both systolic blood pressure and brain PNMT activity (C_1 region only) are significantly elevated over the WKY controls. These elevations are not accompanied by increased epinephrine levels in brain. (3) PNMT activity in the spinal cord is significantly higher in the SHR than in WKY controls. The most profound differences in enzyme levels are seen in the zona intermedia region of the thoracic spinal cord where the specific activity of the enzyme is approximately 2 to 3 times higher in SHR rats than in WKY controls. (4) Inhibition of PNMT by the compound SKF 64139 results in a significant reduction in blood pressure in addition to a decrease in brain and adrenal enzyme activity in the SHR rat. A significant reduction in enzyme activity is also seen in the spinal cord. (5) Both brain and adrenal PNMT appear to be regulated by the adreno-pituitary axis. Hypophysectomy results in a significant reduction in enzyme levels both in brain and adrenal tissue and supplemental glucocorticoid treatment restores enzyme activity to control levels. (6) Chronic treatment with the antihypertensive compound clonidine, results in a

reduction in systolic blood pressure without a corresponding reduction in either brain or adrenal enzyme activity.

Significance to Biomedical Research and Institute Programs: This project is designed to further understand some of the underlying factors that are involved in the initiation of hypertension. It may also permit an evaluation of the role of epinephrine in the regulation of blood pressure by the central nervous system.

Proposed Course of Project:

1. The further examination of regulation of brain and adrenal PNMT.
2. Isolation of a pure enzyme from brain for further determination of regulatory as well as inhibitory properties.

Publications:

Phyall, Willa and Lovenberg, W.: The effects of hypophysectomy on the kinetics and regulation of PNMT in the brain and adrenal glands of rats. In: Catecholamines: Basic and Clinical Frontiers, Usdin, E., Kopin, I. and Barchas, J. (eds.) Pergamon Press, N.Y. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01877-02 HE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Comparison of Molecular Kinetic and Regulatory Properties of Brain and Mast Cell Tryptophan Hydroxylase (Revised Title)

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

PI:	Donald M. Kuhn	Staff Fellow	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem.Pharmacol.	HE NHLBI
	Robert C. Rosenberg	Staff Fellow	HE NHLBI
	Mary Anne Meyer	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:
0.7	0.4	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 molecular parameters of tryptophan hydroxylase from rat brainstem and murine mast cell, were determined by gel filtration and sucrose density gradient ultracentrifugation. The enzyme from rat brainstems has a calculated molecular weight of 220,000 daltons, a Stokes radius of 55.6Å, a frictional ratio of 1.28, and a sedimentation coefficient of 9.63S. The mast cell enzyme has a molecular weight of 144,000 daltons, a Stokes ratius of 50.3Å, a frictional ratio of 1.35, and a sedimentation coefficient of 6.97S. Evidence for catalytically active subunits was not found. The regulatory and kinetic properties of the brainstem and mast cell hydroxylase were also compared. The brain enzyme can be activated by calcium, SDS, trypsin, phospholipids, protein phosphorylation, and by heparin. Of these treatments only heparin activated the mast cell tryptophan hydroxylase. It appears that the tryptophan hydroxylase species from rat brainstem and murine mast cell represent distinct molecular entities.

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Objectives: Tryptophan hydroxylase is the initial and rate-limiting enzyme in the biosynthesis of the neurotransmitter serotonin. Despite numerous studies on the mechanisms by which the activity of this important enzyme is regulated, little is known about the biophysical properties of tryptophan hydroxylase. The objectives of these experiments are to determine some of the molecular, kinetic and regulatory parameters of tryptophan hydroxylase from rat brainstem and murine mast cell, two tissues rich in this enzyme activity.

Methods: Male Sprague-Dawley rats were sacrificed by decapitation and brainstems were rapidly dissected from the brains and frozen on dry ice, and stored in liquid N_2 until assay. Mast cells were obtained from the ascitic fluid of BALB/cX DBA^2 mice as described by Lovenberg et al. (Science 155:217-220,1967). Cells were harvested, washed and frozen in N_2 until used for assay. Tryptophan hydroxylase was assayed by the methods reported by Baumgarten et. al.(J. Neurochem. 21: 251, 1973). Gel filtration (ultragel AcA34) and sedimentation experiments were performed essentially by the methods reported in Rosenberg and Lovenberg (Mol. Pharmacol. 13: 652, 1977).

Major findings: Initial experiments with gel filtration indicated the brainstem tryptophan hydroxylase had a molecular weight of 300,000 while the molecular weight of the mast cell enzyme was estimated to be 200,000. This value for at least the brain enzyme is somewhat higher than the published molecular weight for tryptophan hydroxylase from rabbit midbrain, suggesting that this enzyme may possess an inherent asymmetry. The Stokes radii of the brain and mast cell hydroxylase species as determined by gel filtration were 55.6Å and 50.3Å, respectively. The sedimentation coefficients, determined by sucrose density gradient ultracentrifugation, were 9.63S for the brain enzyme and 6.93S for the mast cell enzyme. Using these values, the molecular weight for the brain hydroxylase was calculated to be 220,000 daltons. The mast cell enzyme has a calculated molecular weight of 144,000 daltons. Calculated frictional ratios were 1.28 for the brain enzyme and 1.35 for the mast cell enzyme, confirming the suggestion that these enzymes are asymmetric in shape.

The kinetic constants (K_m, V_{max}) for the two enzymes were found to be slightly different but of the same order of magnitude. However, the substrate specificities were quite different. The brain enzyme hydroxylates both tryptophan and phenylalanine in a ratio of 13:1 in favor of tryptophan. The mast cell enzyme also hydroxylates both amino acids but at a ratio of only 2:1 in favor of tryptophan.

A comparative study of the effects of a wide variety of substances on tryptophan hydroxylase from the transplantable murine mast cell tumor and rat brain (mesencephalic tegmentum) was also made. Heparin, calcium, limited tryptic proteolysis, sodium dodecyl sulfate, selected phospholipids, and protein phosphorylation each produced activations of tryptophan hydroxylase from rat brain varying from 2 to 5-fold in magnitude. In contrast to these results, most of these same activators either had no effect (trypsin, phosphorylation) or inhibited the activity of the mast cell hydroxylase (sodium dodecyl sulfate, calcium, phospholipids, phosphorylation). Only heparin

activated the mast cell enzyme. The present data taken together with previous results from our laboratory suggest that the tryptophan hydroxylating enzymes from the malignant murine mast cell tumor and rat tegmentum have different molecular, functional, and regulatory properties and may, in fact, be distinct molecular entities.

Significance to Biomedical Research and Institute Programs: These results represent the first systematic determination of the molecular parameters of tryptophan hydroxylase from rat midbrain and murine mast cell. The calculated molecular weight at least of the brain enzyme agrees quite well with the molecular weight of a highly purified tryptophan hydroxylase from rabbit brainstems and, as such, points out the usefulness of these procedures in studying the biophysical properties of a labile enzyme-like tryptophan hydroxylase even in a crude, high speed tissue supernatant fraction. These results also suggest that catalytically active subunits for at least the brain and mast cell enzyme do not exist under the present conditions. Taken together, these data indicate that the tryptophan hydroxylase enzymes from rat midbrain and murine mast cell represent distinct molecular entities. These data also suggest that the determination of the molecular weight of tryptophan hydroxylase by conventional gel filtration methods can lead to errors (over estimations) in estimation due to the inherent asymmetry in this enzyme.

The murine mast cell is a rich source of tryptophan hydroxylase and serotonin. However, very little is known about the mechanisms by which the activity of the mast cell enzyme is regulated (if it is regulated at all). These experiments have shown that the mast cell hydroxylase does not behave like the brain hydroxylase. Additional studies on the molecular mechanisms regulating tryptophan hydroxylase in the mastocytoma cells will help elucidate the means by which this malignant cell line expresses its efficient catalytic activity. These studies also lend valuable information on alternative mechanisms of control of the brain enzyme (e.g., end-product inhibition, allosteric activation).

Proposed Course of Project: Using these same procedures, tryptophan hydroxylase from rat brainstems and murine mast cell will be partially purified so that the regulatory properties of each can be studied in more detail. For example, the effects of calcium, EGTA, thiols, and phosphorylating components in the kinetic properties and substrate specificity of each enzyme will be determined.

Publications:

1. Kuhn, D.M., Rosenberg, R.C. and Lovenberg, W.: Determination of some molecular parameters of tryptophan hydroxylase from rat brainstem and murine mast cell. J. Neurochem., 1979 (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01879-02 HE

PERIOD COVERED
October 1, 1978 to September 30, 1979

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:	H.-Y.T. Yang	Biochemist	LPP NIMH
	W. Lovenberg	Chief, Sect.Biochem.Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

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LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

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0.5	0.5	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Specific immunoglobulins directed toward calmodulin were produced in rabbits. An enzyme-linked immunosorbent assay was developed for the measurement of calmodulin. In striatal supernatant fractions calmodulin exists in free and protein bound form.

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Objectives: Calmodulin, a protein with high affinity binding sites for Ca^{2+} was first described by Cheung and subsequently purifed 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 structure, 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 neuronal elements. There exist experimental evidence that the dopaminergic system in particular is associated with calmodulin. It has been shown that in striatum the dopamine-sensitive adenylate cyclase and cAMP-PDE are regulated by calmodulin. Furthermore, during supersensitivity the calmodulin content in synaptosomal membrane is increased. Since nothing is known on the regulation of calmodulin in the membrane-bound and soluble pool this project will 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: Preparation of antibody directed toward calmodulin. 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.

Enzyme-linked immunosorbent assay (ELISA). 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.

Studies on turnover-rate of calmodulin in striatum. After long-term treatment with haloperidol a cannula was implated 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: Highly specific immunoglobulins directed toward calmodulin showed little cross-reactivity with other species of Ca^{2+} binding proteins such as troponin C or Δ-light chain protein. Calmodulin occurs in high concentrations in the brain where it is particularly enriched in the synaptosomal membrane-fraction (∿ 14 µg/mg protein). In contrast, in adrenal medulla the calmodulin content is 0.40 µg/mg protein and in heart 0.10 µg/mg protein.

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.

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 mechanism of neuroleptic drugs, which act particuarly on the extrapyramidal system, has been shown to be linked to cyclic-nucleotide metabolism, studies on calmodulin-turnover rates will help to reveal some of the molecular features of this action mechanism.

Proposed Course of Project:

1. Studies on the regulation of calmodulin content and translocation of calmodulin from membrane-bound to cytosolic pool will be studied in vivo in regard to response of receptor stimulation including, dopamine, β-adrenergic, gabaminergic and enkephalinergic receptors.

2. In neuroblastoma-glioma cell lines, the molecular mechanism involved in calmodulin synthesis will be studied.

Publications:

1. Hanbauer, I., Gimble, J., Yang, H-Y.T. and Costa, E.: The role of a Ca^{2+}-dependent protein activator purified from brain in the regulation of dopamine receptors. In: Peripheral Dopaminergic Receptors (J.L. Imbs and J. Schwartz,eds.) Pergamon Press, N.Y. and oxford, pp. 289-296, 1979.

2. Hanbauer, I. and Phyall, W.: Involvement of calmodulin in the modulation of dopamine receptor function. In: Long-term of Neuroleptics (Racagni, G., Spano, O., and Cattabeni, F., eds.) Raven Press, N.Y., 1979 (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01880-02 HE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (30 characters or less)

The Role of 3-Deazaadenosine as an Antihypertensive Agent

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

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	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

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LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

3-Deazaadenosine is a compound that has been shown to indirectly cause inhibition of methyltransferase reactions. This compound appears to have immunological as well as antihypertensive potential. As a antihypertensive agent, 3-deazaadenosine has been shown to effectively lower blood pressure in spontaneously hypertensive rats (SHR). The mechanism of the hypotensive effect is not known although the current studies suggest that it may act as a vaso-dilating agent. Because the hypotensive action of this compound is transient, value as a potential antihypertensive agent may be limited.

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Objectives: 3-Deazaadenosine is a metabolic inhibitor which causes the accumulation of S-adenosylhomocysteine and consequently inhibits methylation reactions requiring S-adenosymethionine. The formation of epinephrine is one such system. Since epinephrine neurons are thought to regulate blood pressure it was of interest to determine whether this compound had any effect on blood pressure. Initial experiments indicated that 3-deazaadenosine caused a rapid and dramatic decrease in the blood pressure of both spontaneously hypertensive and control rats. The objectives of this continuing project, therefore, are to determine the mechanism of this hypotensive effect and to evaluate 3-deazaadenosine as a potential antihypertensive agent.

Methods: Male normotensive Wistar-Kyoto and spontaneously hypertensive rats are used in these experiments. Ganglionic blockade was accomplished by the administration of 10 mg/kg hexamethonium 15 min prior to experiments. The cardiovascular (CVR) responses to 3-deazaadenosine will be compared to the CVR responses of various antihypertensive agents by standard techniques. Blood pressure and heart rate are recorded in rats either by the tail cuff technique or via a direct method (arterial cannulation). After 3-deazaadenosine treatment, rats are sacrificed and their brains and adrenal glands removed and assayed for both epinephrine and PNMT concentrations by previously reported methods.

Major Findings: 3-Deazaadenosine significantly lowered blood pressure in both the SHR and normotensive Wistar-Kyoto controls. Following 2 doses of 10 mg/rat the reduction in blood pressure persisted for up to 4 hours. Lower doses were also effective but the duration of action was proportionately shorter. In all cases pressure returned to pretreatment levels and remained stable.

Ganglionic blockade did not affect the hypotensive potency of 3-deazaadenosine suggesting that this compound may have a vasodilatory action. Control experiment with hydralazine, a known vasodilator, gave similar results. Adenosine, itself, is known to be a short acting vasodilator, caused a reduction in blood pressure for 10 min following a single dose. Therefore, 3-deazaadenosine may be acting in a manner analogus to adenosine only with a prolonged time course because of its slower metabolism.

Significance to Biomedical Research and Institute Programs: The role of epinephrine neurons in the CNS regulating blood pressure appears of interest to examine a potential inhibitor of epinephrine synthesis as a blood pressure modulator. 3-Deazaadenosine exhibited powerful hypotensive effects with a rapid onset. While the blood pressure effects are probably unrelated to epinephrine synthesis, other derivatives of adenosine may hold promise as vasodilator antihypertensive agents.

Proposed Course of Project: Experiments are planned which will more clearly define the mechanism by which 3-deazaadenosine exerts its hypotensive effect. The previously planned experiments to determine the effect of 3-deazaadenosine will also be completed. We also plan to explore other derivatives of adenosine as antihypertensive agents.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01881-02 HE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Brain Regional Distribution of Reduced Biopterin

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

PI:	Robert Levine	Chemist	HE NHLBI
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	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHBLI

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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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The investigation of the major determinants of monoamine synthesis and turnover in vivo is of scientific interest because monoamine levels in the central nervous system (CNS) play critical roles in neuropsychiatric, neuroendocrine and cardiovascular diseases. Tyrosine and tryptophan hydroxylase are known to be the rate-limiting steps in the syntheses of catecholamines and serotonin, respectively. Current evidence suggests that the in vivo rate of synthesis of these compounds may be mediated by the concentration of reduced biopterin (BH_4).

Recent reports in the literature have indicated a high correlation between BH_4 levels and tyrosine hydroxylase activity in selected brain areas. Our preliminary results indicate significant amounts of BH_4 in brain areas known to contain large amounts of tyrosine and tryptophan hydroxylase. We have reported that BH_4 content correlates well with both tyrosine and tryptophan hydroxylase activities measured in 10 discrete rat brain areas. An inordinately high content of reduced cofactor is present in the hypothalamus, pituitary, and pineal gland based on the amount of hydroxylase enzyme activity in these same tissues. The presence of unusually high amounts of BH_4 in these "neuroendocrine" type tissues may indicate another role for BH

634

Objectives: In order to examine the potential roles of brain BH_4, it is necessary to determine the levels of this compound in discrete brain areas. In addition, it is of interest to examine both the pharmacologic and physiologic manipulations of BH_4 levels correlated with changes in hydroxylase enzyme activities. Manipulation of BH_4 levels may be more easily accomplished through a detailed understanding of its endogenous biosynthesis.

Methods: Male Sprague-Dawley rats were decapitated and brains were quickly removed in the cold and dissected into the following areas: pons-medulla, striatum, hippocampus, tegmentum, tectum, cerebral cortex, cerebellum, thalamus, hypothalamus, pineal, and septum. In addition, the pituitary retina and adrenals were also dissected. Tissue was stored in liquid nitrogen until time of assay. Tissue samples were homogenized in 1.0 N HCl and aliquots of the supernatant fraction were lyophilized to dryness. Reaction mixture contains .013 units sheep liver quinoid dihydropterin reductase (QDPR), 0.85 µmol NADH, 36 units of highly purified phenylalanine hydroxylase, 10 µmol KPO_4 pH 6.8, 400 units catalase, and 4-^{3}H-L-phenylalanine (30 µC/µmol) in a final volume of 70 µl. Following incubation for 45 min. at 30°C, the reaction mixture is cooled to 0° in an ice bath, and the reaction arrested by addition of 0.1 ml of 1.2 M sodium acetate buffer, pH 5.5. To release any tritium in the 3-or 5-position of the tyrosine formed, 25 µl of N-iodosuccinamine (50 mg/ml in DMSO) is added to the cooled samples. After 5 min the released tritium (as ^{3}HOH) is collected by passage of the reaction mixture over a 0.6 x 3 cm Dowex 50-H + exchange resin. The column is washed twice with 0.70 ml of water, and the total eluent collected in a scintillation vial to which 15 ml of scintillation cocktail is added. A calibration curve using BH_4 standards in 1.0 N HCl is run concomitantly. A UV spectrum is obtained on a 50 µM solution of BH_4 in .01 N HCl just prior to the assay to quantify the concentration of reduced cofactor in the cofactor stock solution.

Major Findings: We have reported significant correlations between BH_4 content and both tyrosine (r=0.64) and tryptophan (r=0.58) hydroxylase enzyme activities across various rat brain areas. The correlation between BH_4 content and total hydroxylase activity (tyrosine + tryptophan) was even better (r=0.80). Our determinations, however, revealed unusually high cofactor concentrations in the hypothalamus, pituitary, and pineal gland when compared to hydroxylase enzyme activity. These areas are known to be involved in many neuroendocrine functions. The high cofactor content in these areas indicates a potential alternative role for BH_4 other than serving as hydroxylase cofactor, possibly related to neuroendocrine function.

Significance to Biomedical Research and Institute Programs: Understanding the levels of BH_4 in discrete brain areas may unveil previously undiscovered physiological roles of this compound.

It is known that patients with Parkinson's disease have significantly lowered BH_4 levels. An understanding of the endogenous biosynthesis of BH_4 may allow for alternative methods for manipulation of BH_4 levels in these patients, possibly improving of their clinical symptoms.

Proposed Course of Project: Further investigation is necessary into determining BH_4 levels in more discrete brain areas as well as other tissues which express high BH_4 content. More sophisticated micro dissecting techniques will be employed. A high pressure liquid chromatographic assay system will be developed for BH_4 measurement to assist us in our studies on BH_4.

In addition, techniques will be developed to study the biosynthetic pathway of BH_4 and the effects of pharmacological agents will be examined as to their effects on the biosynthesis of BH_4.

Publications:

Levine, Robert, Kuhn, Donald and Lovenberg, Walter: Regional distribution of hydroxylase cofactor in rat brain. J. of Neurochem. 32: 1575-1578, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01884-01 HE

PERIOD COVERED October 1, 1978 tc September 30, 1979

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

PI:	Ingeborg Hanbauer	Pharmacologist	HE NHLBI
OTHER:	W. Gaida	Guest Worker	HE NHLBI
	H-Y.T. Yang	Biochemist	LLP NIMH

COOPERATING UNITS (if any)

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Washington, D.C.

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

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)

Opiate-like peptides were isolated from beef adrenal medulla. These peptides show met-enkephalin-like immunoreactivity, but have a higher molecular weight than met-enkephalin suggesting the existence of a immunoreactive precursor. Met-enkephalin and its analogs, Tyr-D-Ala-Gly-Phe-Met-Nltz and FX 33-824 CH decreased the mean arterial blood pressure (MAP) in rats, and this effect could be blocked by naltrexone. In contrast, the reduction of the MAP elicited by β-lipotropin 60-65, β-endorphine and gly-gly-phe-met was not curtailed by naltrexone. The met-enkephalin-like peptide isolated from beef adrenal medulla also decreases MAP. The natural peptide is considerably more potent than the above analogs of met-enkephalin.

627

Objectives: Recent reports suggest that met-enkephalin-like peptides may function as neurotransmitters or neuromodulators in sympathetic ganglia (DiGuidio, A.M. et al., Neuropharmacology 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, when released from afferent axons, may act on opiate receptor 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 action of morphine, therefore, invites to speculate that opiate-like peptide hormones were to be secreted from sympathetic nerves or chromaffin granules, they 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 changes in arterial blood pressure elicited by met-enkephalin, various analogs and met-enkephalin-like peptides isolated from beef adrenal medulla.

Methods: Isolation of met-enkaphalin-like peptides. Met-enkephalin-like peptides were extracted from beef adrenal medulla in 1N CH_3COOH. The homogenate was heat inactivated (5 min at 100°C) and centrifuged at 15,000 x g for 30 min. The supernatant fraction was consecutively chromatographed on Biogel P-2 and P-30 and the different fractions were examined for their met-enkephalin-like immunoreactivity.

Blood pressure measurements . Rats were anesthetized with Choropent (Ford Dodge Lab., 3.0 ml/kg, i.p.) and cannulas were implanted into the jugular vein and carotid artery. The mean arterial blood pressure (MAP) was studied in normal, adrenal demedullated and splanchnicotomized rats. As reference compounds were used: Met-enkephalin, leu-enkephalin, β-lipotropin 60-65, Tyr-D-Ala-Gly-Phe-Met-NH_3 and β-endorphine obtained from Peninsula, California. FK 33-824 CH was obtained from Sandoz, Switzerland.

Major Findings: The MAP was decreased in normal rats after intravenous injection of met-enkephalin (150µg/kg), Leu-enkephalin (150 µg/kg) β-lipotropin 60-65 (150 µg/kg), Tyr-D-Ala-Gly-Phe-Met-NH_3 (30 µg/kg) FK 33-824 CH (1 µg/kg), β-endorphine (150 µg/kg) and Gly-Gly-Phe-Met (100 µg/kg). Pretreatment with naltrexone (1 to 3 mg/kg) curtailed the decrease in MAP elicited by met-enkephalin, IK 33-824 CH and Tyr-d-Ala-Gly-Phe-Met-NH_3, but not by β-lipotropin 60-65, β-endorphine or Gly-Gly-Phe-Met. Met-enkephalin failed to decrease the MAP in adrenal demedullated or splanchnicotomized rats, in contrast--β-lipotropin 60-65 decreased the MAP also in these rats.

Effect of met-enkephalin-like peptides isolated from beef adrenal medulla on MAP . Gel filtration chromatography revealed the existence of at least 3 different forms of met-enkephalin-like immunoreactive substances (A,B, and C). A and B consist of high molecular weight material, whereas C resembles met-enkephalin compounds A,B, and C decreased the MAP in normal rats. The

concentrations required to elicit a significant reduction in MAP were about 10^3 times lower than that for met-enkephalin, when expressed as met-enkephalin-like immunoreactivity. The decreasing effect of compound C appeared to be enhanced in adrenal demedullated rats. Whereas, compound A had no effect on MAP in splanchnicotomized rats.

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 value are always showing respiratory- and vasodepressant effects and since the injection of morphine increases the content of met-enkephalin-like peptides in venous blood it appears important to reveal the action mechanism of these peptides on the cardiorespiratory system.

Proposed Course of Project: Future experiments are planned to reveal (1) whether met-enkephalin-like peptides can be released from adrenal chromaffin cells into adrenal venous blood and (2) on the nature of the receptor regulating this release.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01885-01 HE

PERIOD COVERED October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Substance P Neurons in the CNS

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

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OTHER:	Gary Yurow	Guest Worker	HE NHLBI
	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	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 specific radioimmunoassay (RIA) for substance P (SP) is being used to assign physiological roles to this neuropeptide in specific neurological systems.

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Objective: Substance P (SP) is postulated to be a neurotransmitter in the CNS. In order to study this putative role and its physiological significance, two areas of high SP concentration (i.e., extrapyramidal and nociceptive systems) are neurochemically manipulated by pharmacologic agents and then assayed for possible changes in SP levels.

Major Findings: Chronic treatment with a dopamine (DA) receptor antagonist (haloperidol) reduced SP levels in substantia nigra by 30%, while increasing SP concentration in the periaqueductal grey area by as much as 100%. These results suggest that in some areas of the brain DA helps to regulate the SP system.

In contrast neither chronic nor acute morphine administration had any appreciable effect on SP concentration in brain areas involved in nociception. This suggests that SP does not help mediate the opioid analgesic effect.

The metabolism of SP is little understood, however, peptidase activity is thought to be responsible for its inactivation as a neurotransmitter. We have shown that intraventricular injections of SQ 20881, a peptidase inhibitor, causes significant increases in levels of SP in all brain areas examined. We are examining the possibility of using SQ 20881 as a pharmacological tool to probe SP function.

Significance to Biomedical Research and Institute Programs: SP has been suggested to be important in the function of some neuronal systems. Thus, results indicate that (1) due to its excitatory influence on DA perikarya in substantia nigra, SP helps to modulate locomotor function and (2) SP is involved in sensory (particularly pain) transmission.

Little is known about the interaction of SP with other transmitters involved in locomotor and nociceptive function. Even less is known about the response of SP neurons to drugs which exert pharmacological influences on these neuronal systems. A better understanding of the involvement of SP in these complex neuronal networks would help to assign physiological and pathological roles to this neuropeptide and perhaps suggest new therapeutic approaches for the treatment of related neurological disorders.

Proposed Course of Project: The following objectives will be examined in the coming year.

1. Measure changes in substance P levels that result from stimulation or inhibition of neuronal components of the nigral-striatal system.

2. Attempt to develop a technique for measuring the turnover of substance P.

3. Attempt to elevate substance P in a variety of brain regions by a series of protease inhibitors.

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PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Membrane Phosphorylation in Morphine Tolerant Animals

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

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COOPERATING UNITS (if any)
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LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The endogenous phosphorylation of synaptic plasma membrane (SPM) and synaptosome (SYN) fractions of mouse brain was assayed in vitro using $AT^{32}P$ and $^{32}P_i$ as phosphate donors, respectively. Specific protein phosphorylation was analyzed by autoradiography following SDS polyacrylamide gel electrophoresis. Addition of 50-300 μM Ca^{++} or heated SYN cytosol caused a concentration related increase in the phosphorylation of SPM proteins with apparent molecular weights of 15, 52,59, and 89 K daltons. The phosphorylation of SYN proteins with apparent molecular weights of 30,46,78 and 89 K daltons was increased when this fraction was incubated in the presence of Ca^{++} or Ca^{++} plus the Ca^{++} ionophore, A23187. The effects of Ca^{++}, heated cytosol and A23187 on specific protein phosphorylation were markedly enhanced when the SPM and SYN fractions were obtained from mice rendered tolerant to and dependent on morphine. In contrast, the phosphorylation of the same proteins was reduced when the SPM and SYN fractions were derived from mice undergoing naloxone-precipitated withdrawal.

PHS-6040
(Rev. 10-76)

Objectives: The biochemical signal generated by the binding of an opiate ligand to its specific receptor in neuronal membranes is translated into biochemical actions that alter neuronal function via effector systems. Since protein kinases and phosphoprotein phosphatases are localized in high concentration in synaptic membranes and since specific phosphorylated proteins in the central nervous system may act as physiological effectors for a variety of regulatory agents, it is possible that membrane-bound phosphorylation systems may serve as mediators of the effects of endogenous and exogenous opiates.

The specific objectives of the present investigation were to evaluate the effects of the chronic administration of morphine, the classic opiate analgesic, on the phosphorylation of specific synaptic membrane proteins obtained from synaptosomes or synaptic plasma membrane (SPM) fractions of mouse brain. Recently, it has been established that calcium ion regulates the phosphorylation of specific synaptic proteins, possibly through the involvement of the calcium binding protein, calmodulin. Furthermore, alterations in brain levels of calcium as well as the binding of calcium to brain membranes may mediate some of the acute and chronic effects of opiates. Therefore, most of our present efforts have been confined to an investigation of the effects of opiates on calcium-regulated phosphorylation of specific synaptic membrane proteins.

Methods: Subjects. The subjects were male SW mice that had been rendered tolerant to and dependent on morphine by the implantation of a single pellet containing 75 mg of morphine base. Control subjects received placebo pellets.

Tissue preparation. Mice were decapitated and whole brains (less cerebellum) were removed for tissue preparation. A crude P_2 fraction was prepared by standard techniques. Synaptosomes were prepared from a washed P_2 fraction by flotation on a discontinous 7.5/14% ficoll gradient. Two SPM fractions were prepared from lysed synaptosome by subfractionation on a discontinous 20,25,30,35% sucrose gradient.

Phosphorylation assays; SPM. The SPM fractions (25-100 µg protein) were preincubated for 1 min at 30°C in a mixture containing a final concentration of 50 mM HEPES, pH 7.0, 10 mM $MgCl_2$ and 1 mM DTT. The phosphorylation reaction was initiated with the addition of 1 nmole γ^{32} P ATP. After 15 sec the reaction was terminated and membrane proteins were solubilized by the addition of a "stop solution" containing 9% sodium dodecyl sulfate (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.

Phosphorylation assay; synaptosomes. Synaptosomes (500 µg protein) were incubated at 30°C in Krebs buffer containing 0.32 M sucrose but without calcium and were phosphorylated by exposure to 0.25 mCi P_i for 45 min. The phosphorylated synaptosomes were then transferred to a post-incubation Krebs buffer containing no calcium, calcium or calcium plus the calcium ionophore, A23187. Reactions were terminated at various intervals with the SDS "stop solution". All other procedures were as described for the experiments with the SPM fractions.

Gel electrophoresis. The solubilized SPM and synaptosomal proteins were resolved on SDS polyacrylamide slab gels. The acrylamide concentration was 10% and SDS was included in the resolving and stacking gels at a final concentration of 0.1%. Electrophoresis was performed at constant power (7.5 W/gel). Following electrophoresis the gets were fixed and stained for protein with 0.1% Coomassie brilliant Blue R250 in 50% methanol-10% acetic acid. The gels were destained by diffusion in 30% methanol-10% acetic acid before drying under heat and vacuum.

Autoradiography. Dried gels were placed in close contact with Kodak RP X-ray film which was developed 2 to 4 days later.

Quantitative determination of incorporated phosphate. Autoradiographs were scanned with a Zenith soft laser microdensitometer. The incorporation of phosphate into specific protein bands was quantified from the densitometric scans of the autoradiographs by measurement of the peak areas corresponding to specific protein bands. The background darkness of the autoradiographs was taken as the baseline value for these determinations.

Calcium uptake. The uptake of calcium ion into synaptosomes during the post-incubation periods was measured by spiking the mixture with trace amounts of $^{45}Ca^{++}$. Following exposure to the radioactive calcium, the synaptosomes were filtered and washed. The radioactivity remaining on the washed filters was quantified by liquid scintillation spectrometry.

Major Findings: SPM: The electrophoretic resolution of the SPM proteins was such that at least 40 distinct proteins could be detected by protein staining. Autoradiography revealed that phosphate was incorporated into several protein bands. The most highly phosphorylated bands were those with apparent molecular weights of 15,52,59 and 89 K daltons. In the absence of calcium, the incorporation of phosphate into membrane protein did not differ between the fraction derived from the morphine and placebo subjects. The phosphorylation of the 15,52,59 and 89 K bands was enhanced in the presence of 100-300 μM Ca^{++} or heated synaptosomal cytosol, however, these effects were greater when the SPM proteins were derived from mice implanted with morphine pellets. The administration of naloxone 1 mg/kg to pellet implanted mice resulted in a reversal of the effects due to morphine pellet implantation alone. The qualitative observations from the autoradiographs were confirmed by quantitative densitometry.

Synaptosomes: The autoradiographs of the phosphorylated synaptosomal proteins were qualitatively similar to those obtained with the SPM fraction. However, in this instance, the most highly phosphorylated proteins had apparent molecular weights of 30,46,78 and 89 K daltons. Synaptosomes incubated in the absence of calcium exhibited protein phosphorylation patterns that were identical in both morphine and placebo-treated subjects. An enhanced phosphorylation of the 78 and 89 K dalton bands was observed when Ca^{++} was included in the post-incubation buffer. The phosphorylation of these calcium-regulated bands was always greater when the synaptosomes were obtained from the mice implanted with morphine pellets, an effect that was reversible

by naloxone administered in vivo 72 hours after pellet implantation. The calcium ionophore, A23187, enhanced the phosphorylation of the calcium regulated bands, but always to a greater degree for the proteins derived from the mice implanted with morphine pellets, an effect that was also naloxone-reversible. The effects of the chronic administration of morphine on the phorphorylation of synaptosomal proteins was not due to changes in calcium uptake since calcium uptake in the presence or absence of A23187 was not affected by the implantation of morphine pellets.

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 molecular mechanism(s) involved in the induction of tolerance and dependence of the specific opiate type. Since the endogenous opieate - like compounds, the endorphins, have been shown to produce tolerance and physical dependence in rodents, the mechanism(s) underlying this phenomena is of great interest from a physiological as well as a pharmacological viewpoint. The phosphorylation of specific endogenous protein substrates represents one molecular mechanism through which numerous regulatory agents elicit specific biological responses. Thus, protein phosphorylation represents one possible locus of opiate action that may result in the long lasting homeostatic changes that we observe in vivo as tolerance and physical dependence.

Proposed Course of Project: Time and dose-effect relationships will be established for the effects of chronic opiate administration on specific protein phosphorylation. The relationship between the effects of opiates on protein phosphorylation and the levels and binding characteristics of the calcium binding protein, calmodulin, will also be examined.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03502-01 HE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Biochemical Events Underlying the Flucuations in cAMP Levels in Rat Pineal

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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Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
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0.6	0.6	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The focus of this project was to describe the sequale of biochemical and molecular events regulating cAMP levels in the rat pineal gland following the termination of β-receptor stimulation. Initial experiments were conducted on fresh pineal glands in culture medium. Results showed an apparent translocation of calmodulin from membrane fraction to the cytosol in response to β-receptor blockade. In cultured cells maximal response (2-fold increase) was within 10 min. Termination of β-receptor stimulation could also be accomplished in vivo by exposing dark-adapted animals to light. Under this paradigm, a number of rapidly occurring events were observed: (1) Two-fold increase in cytosol calmodulin within 1 min and return to control levels within 2 min.; (2) activation of PDE within 1 min and then a decrease in activity below control values at 2 min.; and (3) decrease in cAMP levels at 1 min, then a 2 to 3-fold increase from control levels at 4 and 6 min. Present results outline a number of rapidly and possibly interrelated series of biochemical events in the pineal gland in response to termination of β-receptor stimulation.

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Objectives: The pineal gland offers a unique opportunity to examine the biochemical mechanisms underlying short-term flucutations in cAMP levels by the enzymes and phosphodiesterase (PDE). The present project has focused on the role of PDE and its activation by the small membrane bound protein calmodulin following various treatments of fresh pineal glands in culture medium and use of a dark to light paradigm. Some work has also been done to correlate these changes with changes in levels of cylic nucleotides in the pineal and with changes in adenylate cyclase activity.

Methods: Fresh rat pineal glands are routinely used. Following a period of incubation in culture medium, the glands are homogenized, boiled, centrifuged and supernatant used for analysis of activator protein. Calmodulin is determined by the extent of activation of purified bovine heart PDE. Adenylate cyclase is measured by an enzymatic method in which cAMP is generated *in vitro* and the cAMP so generated is assayed by a double antibody radioimmunoassay. Pineal cyclic nucleotides were measured in animals killed by focused-beam microwave by radioimmunoassay.

Major Findings: Incubation of fresh rat pineal glands in culture medium containing isoproterenol resulted in a transient rise in cytosol calmodulin. Levels had risen and then returned to control values within 1 hr. Subsequent addition of propranolol resulted in a rise in cytosol calmodulin which peaked at 10 min and returned to control values within 20-30 min. To observe this rise it was necessary to "preincubate" the pineal glands for at least 1 hr in the presence of isoproterenol. Also the propranolol-induced rise in cytosol calmodulin was independent of cAMP and cGMP and was more specific for ℓ-propranolol than the d, or dℓ-species. The increase initiated by propranolol apparently did not require the simultaneous presence of isoproterenol.

By the present assay technique, the maximal increase in calmodulin was 2-fold. No changes in calmodulin levels were observed if one analyzed sonicated pineals. Under these conditions all of the membrane-bound calmodulin is released into the cytosol thus masking any observable changes in cytosol content of the activator protein from the membrane fraction to the supernatant.

Under conditions of darkness neuronal activity into the rat pineal gland is quite high. Thus, postsynaptic β-receptor are being continuously stimulated. This situation can be rapidly reversed by exposing the animal to light. Using this experimental paradigm we examined its effect on cAMP and a number of factors which regulate cAMP levels. Our results show that following the exposure of dark adapted animals to light there is a rapid but transient decrease in cAMP levels which return to control values within 2 min. However, at the 4 and 6 min time point cAMP levels increase 3 to 5-fold above control values. PDE flucuations are almost exactly the opposite. While there is an increase in activity at the early time point (15 sec to 1 min) there is a significant decrease at the 2,4, and 6 min time point. An analysis of calmodulin revealed a rapid increase within 15 sec, peaking at 1 min and returning to control values within 4 min.

Significance to Biomedical Research and Institute Programs: Present results support the following mechanism in the regulation of cAMP levels: (1) Reduced receptor activation leads to a translocation of calmodulin from membrane to the cytosol and (2) cytosol calmodulin activates PDE which in turn leads to increased metabolism of cAMP.

The pineal is a useful model for understanding and characterizing the innervation of end-organ from a biochemical standpoint because the innervation is simple, anatomically well characterized, and accessable. Furthermore, the biochemical events occurring after neural stimulation of this system are dramatic. As such this model offers an almost ideal opportunity to characterize pre-and post-synaptic events of neural transduction. Results from analysis of this system might offer insights into the pharmacology and physiology of less well-defined systems in sympathetically innervated as well as into the central nervous system itself.

Proposed Course of Project: In the next few months we plan to (1) delineate further and in a more quantitative manner the flucutations in cAMP, calmodulin and PDE activation; (2) determine the initiating factors at the level of the receptor which lead to the possible translocation of the calmodulin from the membrane to the cytosol and (3) concentrate on the mechanism which leads to the decrease in cytosol calmodulin following its rapid increase.

Publications:

Ratliff, B., Miller, L.P. and Lovenberg, W.: Ornithine decarboxylase: Apparent lack of involvement in the induction of N-acetyltransferase in rat pineal gland. Biochem. Pharmacol. 28: 389-391, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03503-01 HE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Characterization of Cerebral Protein Kinases

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

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	Eleanor Bruckwick	Chemist	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
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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)

A trypsin sensitive kinase has been isolated from rat brain. It phosphorylates histone as substrate in a magnesium dependent reaction. It is stimulated approximately 17-fold by trypsin and is cyclic nucleotide independent. It is also stimulated by a calcium-dependent protease.

Objectives: This enzyme has been characterized according to its trypsin sensitivity. Because it is stimulated by a calcium-dependent protease we want to define the possible role of calcium in its direct activation. This will be done by testing for the presence of calmodulin as an integral part of the enzyme complex.

Methods: The enzyme has been purified from rat brain by a combination of DEAE Sephadex, Sephadex G-150 chromatography and by isoelectric focusing.

Major Findings: A highly purified protein kinase was obtained by the isolation procedure described above. This enzyme, however, exhibited little histone kinase activity unless first activated by a protease. A brief exposure to trypsin resulted in a 17-fold activation of the prokinase. The enzyme could also be activated by exposure to the calcium dependent protease of brain. Either before or after proteolytic activation the enzyme was essentially not stimulated by cyclic nucleotides (cAMP or cGMP) and it exhibited optimal activity at 75 mM Mg^{++}. This concentration exceeds that normally considered to give an optimal Mg^{++}-ATP ratio. In addition to the unusual Mg^{++} requirement, significant stimulation has also been observed with Ca^{++}. Since recent studies on other laboratories have demonstrated that phosphorylase kinase and myosin light chain kinase are markedly stimulated by Ca^{++} and calmodulin it is possible that this kinase may also be partially dependent upon calmodulin. Analyses of the highly purified brain prokinase suggest that it contains significant amounts of calmodulin-like activity.

Significance to Biomedical Research and Institute Programs: This kinase may be important in the regulation of catecholamine synthesis. The enzyme may regulate tyrosine or tryptophan hydroxylase by phosphorylation in a cAMP independent fashion. This reaction could be highly calcium-dependent suggesting a more direct role for the kinase in neuronal transmission and an alternate path for the regulation of tyrosine hydroxylase.

With the isolation of an action form of the enzyme it is now possible to study the effects of drugs on decreasing or increasing its activity.

Proposed Course of Project: The presence of calmodulin in the enzyme is being determined through the assay of the ability of boiled enzyme to activate PDE.

The ability of the kinase to phosphorylate tryptophan and tyrosine hydroxylase will be studied by the addition of either hydroxylase to the purified enzyme in the presence of phosphorylating conditions.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03504-01 HE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Stimulation of Rat Brain Tryptophan Hydroxylase by Polyelectrolytes

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

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	Mary Anne Meyer	Guest Worker	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.4	0.4

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The activity of rat brain tryptophan hydroxylase (TH) is increased 2-fold by heparin and 4-fold by dextran sulfate. Hyaluronic acid, chondroitin sulfate, and dermatan sulfate as well as the unsulfated polymer dextran do not alter hydroxylase activity. Heparin and dextran sulfate decrease the apparent K_m of the enzyme for both substrates $6MPH_4$ and tryptophan as well as increasing the V_{max}. A variety of polyanions (DNA, glycogen, poly-d-and poly-ℓ-glutamic acid) have no effect on TH whereas salts [NaCl, KCl, $(NH_4)_2$ SO_4 and $MgSO_4$] inhibit the enzyme, indicating that the effects of heparin and dextran sulfate on TH are not mediated by increases in ionic strength per se. Several lines of data suggest that TH binds ionically to these polyelectrolytes: (1) The activation produced by heparin and dextran sulfate diminishes as the ionic strength of the assay medium increases, (2) TH binds to heparin substituted Sepharose 4B and is eluted by increasing the ionic strength of the eluant buffer, and (3) large molecular weight dextran sulfate (MW=500,000) dramatically shifts the elution profile of TH from a K_{av} of 0.41 to a K_{av} of 0.10 on a Sepharose/CL-6B column. These data suggest that binding of certain poly-electrolytes to TH induces a conformational change in the enzyme resulting in increased catalytic activity.

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Objectives: The in vitro activity of tryptophan hydroxylase can be increased by a variety of substances including the membrane detergent sodium dodecyl sulfate (SDS), phospholipids (Hamon et al., J. Neurochem. 28: 811, 1977) and ATP-Mg^{++} (Kuhn et al., BBRC 82: 759, 1978). Upon close examination these seemingly different treatments may have a common effect on the enzyme. In fact, insight into the molecular mechanisms by which these treatments alter tryptophan hydroxylase can be gained from the results of experiments on a similar enzyme, tyrosine hydroxylase. For example, heparin, phospholipids, and certain biological membranes stimulate the activity of tyrosine hydroxylase. These structurally unrelated substances are all polyanions which apparently activate tyrosine hydroxylase via salt reversible, "electrostatic" interactions with the enzyme (Katz et al., Biochim. Biophys. Acta. 429: 84, 1976). In an effort to more completely understand how the activity of tryptophan hydroxylase is altered by polyanions, including SDS and phospholipids, the effects of various acidic mucopolysaccharides and the model polyelectrolyte dextran sulfate on tryptophan hydroxylase were investigated.

Methods: Male Sprague Dawley rats were decapitated and the mesencephalic tegmentum was rapidly dissected from the rest of the brain, frozen on dry ice 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).

Affinity chromatography and gel filtration. Columns (0.6 x 3 cm) of heparin and ethanolamine substituted Sepharose 4B were exhaustively washed with 0.05 M Tris HCl (pH 7.4) containing 0.002 M DTT. Approximately 1.2 mg of crude enzyme was passed over either column and the gel was washed with 5 bed volumes of the same buffer. Fractions of 0.05 ml were collected. The column was then eluted with 5 bed volumes of 0.05 M Tris HCl (pH 7.4) containing 1.0 M NaCl and 0.002 M DTT and 0.5 ml fractions were collected. Each fraction was subsequently assayed for tryptophan hydroxylase as described above. Gel filtration was carried out on a column (0.9 x 29 cm) of Sepharose/CL-6B equilibrated with 0.05 M Tris HCl (pH 7.4) containing 0.002 M DTT. The column was pumped in a descending direction at a linear flow rate of 15.5 ml/cm^2-hr and 6 drop (0.38 ml) fractions were collected. The crude enzyme (2.5-3.0 mg protein) was layered over the gel by adding either dextran or dextran sulfate (MW=500,000 for each) to the tissue extract to a concentration of 5.0 mg/ml. All fractions were assayed for tryptophan hydroxylase as described above. The void volume of the Sepharose/CL-6B column was marked with Blue Dextran. All chromatographic procedures were carried out in a cold room (4°C).

Major Findings: Of all the mucopolysaccharides tested, only heparin (1mg/ml) altered enzyme activity (2-fold increase). The model polyanion dextan sulfate produced a 4-fold stimulation of enzymic activity at a concentration of 0.5 mg/ml. The increases produced by heparin and dextran SO_4 were not mediated simply by increases in ionic strength since increasing the ionic strength of the assay medium with various salts produced inhibition of tryptophan hydroxylase. Heparin (1.0 mg/ml) decreased the apparent K_m for $6MPH_4$ from 217 µM to 112 µM. V_{max} was also increased from 0.525 to 0.778 nmol/mg/min. Heparin

had a smaller effect on tryptophan kinetics, decreasing the apparent K_m from 132 μM to 91 μM and increasing V_{max} from 0.50 to 0.77 nmol/mg/min. The effects of dextran sulfate (0.5 mg/ml) on the kinetic properties of tryptophan hydroxylase are qualitatively the same as those of heparin except that its effects on V_{max} are somewhat larger in magnitude (data not shown).

In an effort to determine if tryptophan hydroxylase was interacting directly with the polyanions, the enzyme was chromatographed on a column of heparin-Sepharose (affinity gel). It was observed that tryptophan hydroxylase binds to some extent to the heparin-Sepharose. Approximately 44% of the total recovered activity was bound to the gel. The addition of heparin (2 mg/ml) to either the equilibrating buffer or the enzyme solution prior to chromatography completely blocked the binding of tryptophan hydroxylase to the gel (data not shown). The ionic nature of the binding is indicated by the NaCl (1.0 M) induced elution of the enzyme. Furthermore, if the heparin-Sepharose was equilibrated with buffer containing 1.0 M NaCl, the enzyme did not bind to the column (data not shown). Finally, tryptophan hydroxylase did not bind to ethanolamine treated CnBr-Sepharose 4B. The enzyme-polyelectrolyte interaction was further investigated by gel filtration. It was assumed that if heparin and dextran sulfate were binding to tryptophan hydroxylase, the polyanion-enzyme complex should chromatograph quite differently from the native enzyme. The use of a large molecular weight dextran sulfate (MW = 500,000) made this type of experiment technically feasible. Tryptophan hydroxylase, when chromatographed with dextran (5 mg/ml) emerges in a retained fraction. The peak tube is at fraction 31 which corresponds to a K_{av} of 0.41. However, if dextran sulfate is added to the enzyme solution (5 mg/ml) prior to chromatography, the enzyme is eluted at fraction 22, corresponding to a K_{av} of 0.10. The shoulder on the low molecular weight side of the enzyme + dextran sulfate curve probably represents enzyme activity which was not bound to dextran sulfate. This dramatic shift in the elution profile of the hydroxylase indicates that the enzyme is bound to dextran sulfate.

It is well known that the surface charge potential of high density polyelectrolytes can be compromised by increases in the concentration of a mobile counterion. Based on this fact as well as the observation that NaCl prevents binding of tryptophan hydroxylase to heparin-Sepharose (above), it was predicted that the increase in tryptophan hydroxylase activity produced by either heparin or dextran sulfate would diminish with increase in ionic strength. In support of this prediction, the enzyme activation produced by either heparin or dextran sulfate is completely blocked by NaCl (I = 0.2 M).

Significance to Biomedical Research and Institute Programs: These results indicate that tryptophan hydroxyase, like tyrosine hydroxylase, can be activated by its association with polyanionic molecular species. Heparin and other sulfated mucopolysaccharides are present in and transported along neuronal pathways; therefore, the alteration in tryptophan hydroxylase activity by polyanions may be a likely mode of regulation of this important enzyme in vivo. These results also contribute more generally to knowledge about the electronic and possible conformational requirements for maintaining optimal rates of tryptophan hydroxylation. The recent immunohistochemical

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demonstration of what appears to be an association of tryptophan hydroxylase with subcellular organelles further supports the suggestions that binding of tryptophan hydroxylase to cellular particles (perhaps with net anionic charges) leads to increases in enzyme activity.

Proposed Course of Project: Additional studies will be carried out to identify other cellular polyanionic substances which can activate tryptophan hydroxylase. Advantage will also be taken of the ability of tryptophan hydroxylase to bind to heparin and dextran SO_4 in the purification of the hydroxylase. Finally, conformational changes in the enzyme structure will be studied in more detail (using a purer enzyme preparation) in an effort to assess the extent to which rapid changes in enzyme structure (in response to a variety of stimuli) lead to increases in activity and, in turn, increases in the neurotransmitter serotonin.

Publications:

1. Kuhn, D.M., Meyer, M.A. and Lovenberg, W.: Activation of rat brain tryptophan hydroxylase by polyelectrolytes. Biochem. Pharmacol. 1979 (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03505-01 HE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
The Role of Oxygen, Iron, and Sulfhydryl Groups in the Stablization and Catalytic Activity of Tryptophan Hydroxylase

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

PI:	Donald M. Kuhn	Staff Fellow	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI
	Belle Ruskin	Research Assistant	HE NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.3	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)

Tryptophan hydroxylase appears to be an oxygen sensitive, iron requiring enzyme. Exposure of the crude enzyme to 100% 0_2 for 30 min. at 37°C produces a 70% reduction in activity. Superoxide and hydroxyl radicals are not involved in the inactivation. There is no loss of activity at higher temperatures if the enzyme is kept under N_2. Activity can be recovered by the addition of thiols. Iron chelators can also strongly inhibit tryptophan hydroxylase, at least in part, by competitive inhibition for the substrates 6MPH$_4$ and tryptophan. If the enzyme is exposed to 25°C in room air (20% 0_2) for 24-48 hrs, a substantial loss of activity is observed. Activity can be largely recovered by incubating the enzyme (24 hrs) under anerobic conditions in the presence of dithiethreitol and iron at 25°C. Preliminary experiments have also suggested that the addition of inorganic sulfide produces an additional recovery of activity. Finally, sulfhydryl reagents strongly inhibit tryptophan hydroxylase. Taken together, these data suggest that tryptophan hydroxylase can exist in various activity states depending on the extent of oxidation or reduction of critical SH groups. The enzyme may be an iron-sulfur protein.

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Objectives: Tryptophan hydroxylase is the initial and rate-limiting enzyme in the biosynthesis of the neurotransmitter serotonin. Progress in the determination of the physical and regulatory properties of this important enzyme has been slow, apparently as a result of the extreme lability of tryptophan hydroxylase. The primary objectives of these experiments were to determine the effects of sulfhydryl groups, iron and oxygen on the stability and catalytic activity of tryptophan hydroxylase. It is of particular interest to determine if tryptophan hydroxylase is an iron-sulfur protein.

Methods: Tryptophan hydroxylase was assayed in rat mesencephalic tegmental extracts by the method of Baumbarten et al. (J. Neurochem. 21: 251, 1973). For 0_2 and N_2 studies the enzyme was put into 15 x 100 glass tubes fitted with airtight stoppers. The enzyme solution is gassed through a 21 gauge needle and exhausted through another 21 gauge needle. Both needles were removed after 3 min of gas (0_2 or N_2) exchange.

Major Findings: Exposure of tryptophan hydroxylase to 100% 0_2 at 37°C results in rapid losses of activity. Under these conditions the enzyme has a half-life of 12-14 min. The loss of activity seen was prevented if 0_2 was replaced by N_2 . Superoxide and hydroxyl radicals were not responsible for the loss of activity since the radical scavengers superoxide dismutase, catalase, inosine, mannitol, and histidine did not prevent 0_2 inactivation. The 0_2 induced inactivation could be prevented and reversed by adding dithiothreitol to the enzyme solution.

The iron chelators o-phenanthroline, d,α-dipyridyl, and 8-hydroxyquinoline are potent inhibitors of the hydroxylation reaction. It appears that the inhibition produced by these agents is mediated, at least in part, by competitive inhibition for the cosubstrates $6MPH_4$ and tryptophan. Chromatography of the enzyme on Sephadex G-25 after preincubation with o-phenanthroline does not completely reverse the inhibition, possibly indicating that there is residual o-phenanthroline still bound to the iron in the enzyme. The enzyme gradually recovers activity as the Fe-o-phenanthroline complex dissociates. A more potent and very specific iron chelator, deferoxamine, also inhibits tryptophan hydroxylation.

Reconstitution of enzyme activity has proved to be a more difficult procedure. Thus far, enzyme inactivated by exposure to room air (20% 0_2) at 25°C for 24-48 hrs. has been successfully reconstituted by incubating the enzyme anerobically at 25°C in the presence of 5 mM dithiothreitol and 50 μM ferrous ammonium sulfate for 24 hrs. The highest recovery of activity is obligatory to both substances. In a few experiments the inclusion of inorganic sulfide lead to a slightly greater recovery of activity.

Finally, the sulfhydryl reagents PCMB, iodoacetate, N-ethyl maleimide, and mercuric acetate each inhibited tryptophan hydroxylase. Mercuric acetate was the most potent inhibitor.

Significance to Biomedical Research and Institute Program: These studies have identified a few critical factors which are responsible for stabilization of tryptophan hydroxylase. As a direct result, more diligent attempts to purify the enzyme can now be made. Once accomplished, numerous studies on the physical, kinetic, and regulatory properties of the purfied enzyme can be carried out. These types of studies have not been possible in the past because of the failure of investigators to achieve significant purification, ostensibly as a result of the extreme lability of tryptophan hydroxylase. These experiments have also suggested that tryptophan hydroxylase may be an iron or iron-sulfur protein, a fact which was implied over 15 years ago but not completely verified.

Proposed Course of Project: Experimentation will continue in the area of enzyme stabilization and the possiblity of extraction and purification of tryptophan hydroxylase anerobically is being pursued. Additional kinetic studies are proposed to assess the roles of iron and sulfur in the catalytic activity of tryptophan hydroxylase.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03506-01 HE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Analysis of N-Acetyltransferase in Rat Retina

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

PI:	Leonard Miller	Staff Fellow	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem.Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

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)

The scope of this project was to analyze for the presence of N-acetyltransferase (NAT) in rat retina. Previous investigators had detected in rat retina the enzyme hydroxyindole-O-methyltransferase (HIOMT) and the hormone melatonin. Therefore, to confirm that melatonin could be synthesized in this tissue, it was necessary to confirm the presence of NAT. Present results showed that NAT is present but only at less than 10% of that detected in rat pineal. Maximal activity (204 pmol/retina/10' or 115 pmol/mg protein/10') occurred at midnight. TLC with two different solvent systems confirmed that the product formed from labelled acetyl CoA and 5-hydroxytryptamine was labelled N-acetyltryptamine.

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Objectives: The hydroxyindole pathway is the only known pathway whereby hormone melatonin can be synthesized. The enzymes which make up this pathway are tryptophan hydroxylase (TH), N-acetyltransferase (NAT) and hydroxyindole-O-methyltransferase (HIOMT). While HIOMT and melatonin were shown to be present in the rat retina there was no evidence that either TH or NAT were present. This investigation was therefore initiated to analyze and confirm that NAT was present in the rat retina.

Methods: Levels of the enzyme NAT were measured in individual retina by a sensitive radiochemical technique. TLC labelled product N-acetylserotonin was identified on Silica Gel 60 F-254 (250 μ thick) using two different solvent systems (A) chloroform-methanol $^{-1}$NHOAC 97:3:1 and ethylacetate. All drugs were administered i.p. between 5:30 and 6:00 p.m.

Major Findings: Analysis by TLC using two different solvent samples confirmed that under present assay conditions labelled N-acetylserotonin is formed. This is the product formed by the action of the enzyme NAT on the substrates acetyl CoA and serotonin. Thus present results confirmed the presence of NAT in rat retina. Maximal enzyme activity was observed at midnight. Pretreatment of rats with cycloheximide (20 mg/kg) significantly attenuated this increase while propranolol (20 mg/kg), phenoxybenzamine (20 mg/kg) and serotonin had no effect. PCPA (300 mg/kg) treatment resulted in a slightly greater increase in NAT levels than normal.

Enzyme activity showed some flucuations with maximal activity occurring at midnight.

Significance to Biomedical Research and Institute Programs: NAT present in retinal cells may be part of a hydroxyindole pathway which can synthesize small amounts of melatonin. This is of interest since both the eye and pineal gland are light sensing organs.

Proposed Course of Project: Present studies were performed with the purpose of confirming presence of NAT in retina and at this time will not be pursued.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03507-01 HE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
α-Receptor Binding in Various Areas of Brain from Hypertensive Rats

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

PI:	Leonard Miller	Staff Fellow	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem.Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The purpose of the present project was to probe for the relationship between α-receptors in discrete areas of rat brain and blood pressure regulation. Specially bred WKY,SHR and SHR/SP rats were used for this investigation. The specific areas removed and analyzed were the hypothalamus, hippocampus, an area containing the locus coerulus and the A_2 area containing the nucleus tractus solitarius. The ligands used for the analysis were the antagonist, WB4101 and the agonist clonidine. There were no differences in WB 4101 binding in any of the areas examined. Clonidine binding studies, however, did reverse some differences; significantly decreased binding in the hippocampus, L.C. and A_2 region of the SHR rats compared to control WKY. Only the hippocampus of the SHR/SP showed significantly less binding than control. Present investigations are pursuing the kinetic characteristics of this reduced binding.

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Objectives: The role of the central nervous system in the etiology of essential hypertension is at present unclear. There has, however, been some evidence suggesting a link between the noradrenergic system and hypertension. Over the last few years techniques have been developed whereby one can investigate certain functional characteristics of neuronal systems. One of these is the probing of receptors by the use of specific ligands. The purpose of the present project was to apply this technique to a recently bred hypertensive strain of rats.

Methods: Linearity with respect to protein concentration was confirmed in each region for both WB 4101 and clonidine binding. For the comparative analysis each ligand binding was analyzed at low concentrations (2 mM) while specific binding was displaced using 10 μM phentolamine.

Major Findings: Binding studies using the α-receptor agonist clonidine revealed differences in the extent of binding in three of the four regions examined in the SHR rats. Specifically, binding in the SHR rats was reduced compared to control WKY in the hippocampus (by 34%) in the area containing the locus coerulus (by 38%) and in the A_2 region (by 27%). However, only the hippocampus of the SHR/SP rats showed significantly decreased levels (by 46%).

Significance to Biomedical Research and Institute Programs: Present data is only preliminary but it is consistent with prior concepts suggesting a decreased efficacy of the central adrenergic system in the spontaneously hypertensive rats.

Proposed Course of Project: Perform a kinetic analysis to determine if the changes are in dissociation constant or in receptor density.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03508-01 HE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Regulation of Sympathetic Activity in the Rat Pineal

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

PI:	Larry Alphs	Research Associate	HE NHLBI
OTHER:	Walter Lovenberg	Head, 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:
0.5	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)

Pineal acetyl coenzyme A:arylamine-N-acetyltransferase (NAT) activity was measured after pretreatment of rats with a variety of putative transmitters and their antagonists. Pretreatment with the α-adrengic agents phenoxybenzamine and phentolamine prevented the rapid light-mediated decrease in NAT activity, while pretreatment with yohimbine was ineffective in altering this phenomenon. When animals were pretreated with intraventricular injections of either clonidine or norepinephrine, a decrease in pineal NAT activity was observed, mimicking the effects of light. Other adrenergic agonists were ineffective in lowering NAT activity and norepinephrine was ineffective in lowering NAT activity when administered peripherally. Phenoxybenzamine was ineffective in blocking the decrease in NAT activity observed after administration of clonidine. However, yohimbine prevented the decrease observed after pretreatment with either clonidine or norepinephrine. These results suggest that central α-receptors play a role in the modulation of NAT activity in the rat pineal.

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Objectives: The goal of this work was to characterize some of the mechanisms involved in the regulation of the light-mediated decrease in pineal NAT activity.

Methods: Rats were maintained on a 12 hour light-12 hour dark schedule for at least one week prior to drug administration. Animals were sacrificed 4-8 hours into the dark portion of their lighting schedule. NAT was measured by radioenzymatic method of Klein and Weller (Science, 169: 1093-1095, 1970).

Major Findings: (1) Phenoxybenzamine and phentolamine prevented the rapid light-mediated decrease in pineal NAT activity, while yohimbine was without effect. (2) Intraperitoneal administration of clonidine, an α-adrenergic agonist, produced a rapid decline in elevated levels of pineal NAT activity. (3) Peripheral administration of other α-adrenergic agonists including norepinephrine, phenylephrine, metholamine and isoproterenol did not effect changes in elevated levels of pineal NAT activity. (4) Intraventricular administration of clonidine and norepinephrine precipitated decreases in pineal NAT activity while other α-agonists remained ineffective. (5) Phenoxybenzamine was ineffective in preventing the clonidine mediated decrease in pineal NAT activity whereas pretreatment with yohimbine prevented the decrease. (6) When animals were simultaneously exposed to light and administered clonidine, pretreatment with both phenoxybenzamine and yohimbine were necessary to prevent a decrease in pineal NAT activity.

These results suggest that receptors with α-adrenergic properties are involved in the regulation of the decrease in pineal NAT activity when rats are exposed to light. Our data suggest that these receptors are centrally located and that there may be two distinct populations of receptors modulating this decrease in enzyme activity.

Significance to Biomedical Research and Institute Programs: This project uses the pineal as a model system for understanding sympathetic neuroregulation of biochemical events in a peripheral organ system. Because the changes in enzyme activity are both rapid and marked this seems to be an excellent model for understanding central and peripheral regulation of sympathetic activity generally. Because of similar ties with central regulation of blood pressure, essential hypertension is a disease which might be more clearly understood by means of this model. Of course, these experiments may also lead to a clearer elucidation of the function and role of the pineal gland itself.

Proposed Course of Project: Future experiments are planned to elucidate the role of cyclic nucleotides in the regulation of pineal NAT activity. Also, experiments are planned using preganglionic stimulation of the sympathetic innervation to the pineal to observe changes in cyclic nucleotides, tyrosine hydroxylase and NAT activity.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03509-01 HE

PERIOD COVERED
October 1, 1979 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Cyclic AMP Specific, Calcium Independent Phosphodiesterase from a Murine Mastocytoma

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

PI:	Krishnamoorthy Sankaran	Visiting Fellow	HE NHLBI
OTHER:	Donald Kuhn	Staff Fellow	HE NHLBI
	Walter Lovenberg	Chief, Sect. Biochem Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

NONE

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A cyclic AMP specific phosphodiesterase has been identified in a malignant tumor (P 815) of murine mast cells. The PDE is found primarily (85%) in the soluble fraction of the cell. The enzyme, purified approximately 10-fold by gel filtration, occurs in a single molecular and kinetic form (low K_m), and is apparently not dependent on calcium and calmodulin for optimal activity. Although cGMP is hydrolyzed at only 4% of the rate of cAMP hydrolysis, this cyclic nucleotide inhibits cAMP PDE activity by 50-60% at a concentration 2.5 μM. The mast cell PDE activity also displays anomalous behavior on gel filtration and sucrose gradient centrifugation. The Stokes radius was determined by gel filtration to be 54.4 Å and the $S_{20,w}$ was determined by gradient centrifugation to be 2.73S. Using these values a molecular weight of 61,000 and a frictional ratio of 1.93 were calculated. This PDE is apparently an asymmetric enzyme with novel molecular and regulatory properties.

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PHS-6040
(Rev. 10-76)

Objectives: Multiple forms of cyclic nucleotide phosphodiesterase (PDE) have been identified in extracts of many mammalian tissues. These forms differ in their kinetic behavior, substrate specificity and response to various effectors and seem to exist in different ratios in various type cells. At least one form of these phosphodiesterases can be activated by calmodulin, an endogenous thermostable protein. Interaction of this enzyme with calmodulin and calcium results in a decrease in the K_m of the enzyme for substrate and an increase in V_{max}. However, a complete definition of the elements involved in the regulation of these various forms has not been achieved.

Intracellular concentrations of cyclic nucleotides are thought to influence cell proliferation and maturation. Furthermore, the cAMP levels in the corresponding normal tissues and the in vivo administration of cAMP or theophylline causes retardation of tumor growth. Taken together, these data suggest that the proliferation of some malignant tumors is associated with lowered cAMP levels which can be explained, at least in part, by increased activity of the cAMP degradative enzyme PDE. With these views in mind, a study was undertaken to evaluate the nature of cyclic nucleotide phosphodiesterases in malignant murine mast cells. Specifically, we sought to determine some molecular (molecular weight, Stokes radius, frictional ratio, sedimentation coefficient) and regulatory properties (substrate specificity and inhibition, calcium dependence) of the mast cell PDE.

Methods: Male Balb/c x DBA/F, hybrid mice (20g) were obtained from Charles River Laboratories. Neoplastic (P815) murine mast cells were harvested and transplanted as previously described by Levine, Lovenberg, and Sjoerdsma (Biochem. Pharmacol. 1964, 13 1283-1290). [^{3}H]cAMP (specific radioactivity, 39.8 ci/mmol) and [^{3}H]cGMP (specific radioactivity, 8128 Ci/mmol) were purchased from New England Nuclear Corporation (Boston, MA). cAMP, cGMP and dithiothreitol were purchased from Calbiochem (San Diego, CA). Snake venom (Ophiophagust hannah) from Sigma Chemical Company (St. Louis, MO) was used as a source of 5' nucleotidase.

Murine mast cells were thawed and added to an equivalent volume of 0.05 M Tris-CHl, pH 7.4 and ultrasonicated (three 15 seconds intervals at 4^{o}C with a 1 minute interval between each sonication), with a micro-ultrasonic cell disruptor. The cell sonicates were centrifuged at 40,000 x g for 20 minutes at 4^{o}C and the supernatant fraction was used for further studies. Phosphodiesterase activity was measured by the isotopic method of Filburn and Karn (Analytical Biochem., 1973, 52, 505-516). Protein was estimated spectrophotometrically at 280 nm by the method of Lowry et al., (J. Biol Chem., 1951, 193, 265-275) or by the use of the Biorad protein assay kit.

Major Findings: The 40,000 x g supernatant of murine mast cell was tested for PDE activity with cAMP and cGMP as substrates. At the concentration of cyclic nucleotides (6.25 µM) presently used, the specific activity of cAMP PDE (5.41 nmoles/mg of protein 10 minutes) was found to be approximately 10-15 times higher than similarly purified PDE from other sources (e.g. bovine brain and bovine pineal). When the concentration of cAMP in the assay was varied from

1.25 -62.5 μM, the apparent K_m for this substrate was 4 μM. No enzyme activity could be detected with cGMP as substrate at a concentration of 6.25 μM. However, when the cGMP concentration system was lowered to a 1.0 μM, a small amount of cGMP hydrolysis (specific activity 0.21 nmoles/mg of protein) could be seen. After repeated freeze-thawing, the majority (about 85%) of the PDE activity for cAMP was found in the soluble fraction of the cell. The enzyme activity (12-15%) remaining in the particulate fraction could be released by sonication. The particulate fraction also had little if any cGMP hydrolyzing activity.

When the 40,000 g supernatant fraction was chromatographed on a Sepharose 4B column the fractions possessing the enzyme activity with cAMP emerged as a single peak. No enzyme activity could be seen with cGMP in any of the fractions. The enzyme protein was resolved from the major non-PDE protein peaks, eluting near the position where ferritin emerged from the column under similar conditions. This gel filtration step produced an approximate 10-fold increase in specific activity. Like most other cyclic nucleotide PDEs, mast cell PDE has an absolute requirement for divalent Mg^{++} ions for enzyme activity (i.e., EDTA completely inhibits PDE activity). Dithiothreitol or Ca^{++} had no effect on the enzyme activity. The addition of calmodulin to the assay system did not stimulate the enzyme activity either in the presence or absence of Ca^{++}. EGTA at a concentration of 1 mM did not inhibit the basal activity of the enzyme.

The effects of varying concentrations of cGMP on the hydrolysis of cAMP by mast cell PDE were also investigated. At lower concentrations of cGMP (1-10 μM), the inhibition of enzyme activity was 25-40%. The inhibition of cGMP at a concentration of 25 μM was 56-60% and remained the same even at concentrations of cGMP up to 100 μM. As expected, aminophylline, theophylline, and caffeine, at concentrations of 5 mM each, produced 90-100% inhibition of PDE activity.

The results of gel filtration and sucrose density gradient centrifugation yielded quite discrepant results. The apparent molecular weight of the PDE was approximately 390,000 based solely on Sepharose/CL-6B gel filtration. However, the enzyme behaved much like ovalbumin ($s_{20,w}$=3.6) on sucrose gradients. The sedimentation coefficient of the single peak of PDE activity was estimated to be 2.73 $\pm$ .15 (N=7) by comparing its sedimentation position to that of the standard proteins. Using the expression $S_1/S_2=(MW_1/MW_2)$, where S_1 and S_2 refer to sedimentation coefficients of the PDE and ovalbumin respectively, the MW of the mast cell enzyme was calculated to be only 28,400. This large discrepancy in the MW estimations by gel filtration and sucrose gradient centrifugation suggests that the enzyme may possess an inherent asymmetry in its molecular shape. A more realistic estimation of the MW of this PDE can be obtained by combining data from gel filtration (specifically, Stokes radius) and sedimentation ($s_{20,w}$) experiments. The Stokes radius of the mast cell PDE was 54.4 $\pm$ 1.3Å (N=3), as determined by gel filtration. Using the independent determined $s_{20,w}$ (2.73) and Stokes radius (54.4), the MW was calculated to be 61,000 and the frictional ratio (f/f_o) was calculated to be 1.93. This large f/f_o supports the presumption that the mast cell PDE is quite an asymmetric protein.

Significance to Biomedical and Research Institute Programs: The foregoing results with the mast cell PDE are significant in that they represent the first

demonstration of a PDE enzyme which exists in a tissue as a single molecular and kinetic (high affinity) form. Furthermore, the enzyme is substrate specific for cAMP and is apparently not dependent on either calcium or calmodulin for optimal activity. Additional studies are necessary to determine if this PDE is the prototypical catalytic unit of many other PDE enzymes.

Preliminary experiments with a boiled mast cell PDE preparation revealed that the extract could activate PDE from bovine pineal in a calcium dependent manner. Furthermore, this activating property chromatographed the same as the PDE activity before boiling. These results, along with the observation that Ca^{++} and calmodulin do not stimulate basal PDE activity (above), suggest that calmodulin may be tightly bound to the PDE. It is also possible that calmodulin may be a subunit of this particular PDE much like it is with phosphorylase kinase.

It has been suggested that malignancy is often associated with low effective levels of cAMP. The cytosol fractions of hepatoma cells are deficient in cAMP binding sites and a shift in the elution profile of liver protein kinases, the activities of which were associated with cAMP were reported in Yoshida ascites tumor bearing rats. The high relative specific activity of PDE, resulting in low levels of cAMP (preliminary experiments revealed that the concentration of cAMP in cell extracts is 5.3 nM and the concentration of cGMP is 5.2 nM), may therefore have some bearing on various aspects of altered cellular metabolism in the malignant mast cells.

Proposed Course of Project: Studies are underway to purify the mast cell of PDE to homogeneity. Once purified, more direct physical methods will be used to determine molecular weight and other properties of the PDE. In addition, attemps will also be made to produce antibodies to the purified PDE.

In an attempt to learn more about the relationship of cellular malignancy to the activation of PDE, cells will be assayed for PDE at various stages of malignancy so that a correlation can be made between total enzyme activity and altered cellular proliferation. With antibodies, it can be determined whether the enzyme is activated or if additional enzyme molecules have been synthesized. Finally, attempts will be made to ascertain if calmodulin is in fact a subunit of the mast cell PDE.

Publications:

1. Sankaran, K., Kuhn, D.M., and Lovenberg, W.: Cyclic AMP specific, calcium independent phosphodiesterases from a malignant murine mast cell tumor. Biochem. Biophys. Res. Commun., 1979, (In press).

ANNUAL REPORT OF THE
LABORATORY OF KIDNEY AND ELECTROLYTE METABOLISM
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
OCTOBER 1, 1978 THROUGH SEPTEMBER 30, 1979

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 of 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 a 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. Transport of calcium by cortical thick ascending limbs of Henle's loop. The thick ascending limb was previously identified to be a major site of control of calcium reabsorption. Bourdeau and Burg have been measuring calcium fluxes in this segment. They previously found that calcium transport was passive, driven by transepithelial voltage (which normally is positive in the lumen). Also, at high positive voltages parathyroid hormone and analogues of cyclic AMP greatly stimulated calcium absorption. The purpose of the studies during the past year was to investigate the mechanism of this hormone action. When the transepithelial voltage was altered experimentally, parathyroid hormone caused calcium absorption to increase at positive voltages, caused no change in calcium transport (which remained zero) at zero volts, and caused the calcium secretion that occurs at negative voltages to decrease. Thus the hormone rectified calcium transport in a lumen to bath direction. It remains to be seen whether there actually is a rectifier in the cell membranes that is affected by the hormone or whether there is some other explanation for the results.

2. CO_2 permeability of rabbit proximal convoluted tubules. Because of the importance of CO_2 in urinary acidification, Schwartz, Weinstein, Steele, Stephenson, and Burg have measured transepithelial CO_2 permeability directly in isolated perfused tubules.

The luminal and peritubular unstirred layers were found to provide approximately half of the total tissue resistance to CO_2 permeation. The mean epithelial CO_2 permeability was very high, $12X10^{-5}$ cm^2/s. That corresponds to a cellular diffusion coefficient for CO_2 one-half as great as in free solution. The diffusion of CO_2 within the cells probably is facilitated by carbonic anhydrase-dependent reactions, since the carbonic anhydrase inhibitor acetazolamide reduced CO_2 permeability by half. The CO_2 permeability is so high that bicarbonate absorption by rabbit tubules should not result in any substantial transepithelial CO_2 pressure difference, even if reabsorption involves reaction of all the absorbed bicarbonate with secreted protons to form CO_2 in the lumen.

3. Control of sodium reabsorption by renal tubules. Knepper and Burg have investigated the sites and mechanisms of control of sodium reabsorption in the nephron. Previous studies demonstrated mineralocorticoid stimulation of sodium transport in cortical collecting ducts. Those results fitted the generally accepted theory that mineralocorticoids such as aldosterone directly stimulate sodium reabsorption in "distal tubules" but not "proximal tubules." During the past year the second part of the theory, i.e. no "proximal" effect, has been tested. Surprisingly, chronic administration of desoxycorticosterone to rabbits greatly increased sodium and fluid absorption by their proximal straight tubules in vitro which, taken at face value, is against the theory. In addition, the tubule cells hypertrophied visibly. While this may represent an unexpected mineralocorticoid effect on proximal tubules, other possibilities are being investigated. For example, the effect might be glucocorticoid since, at the high doses used, desoxycorticosterone has glucocorticoid, as well as mineralocorticoid action. Also, the effect might be a generalized trophic change in the kidney, associated with increased glomerular filtration rate or extracellular fluid volume.

4. Acidification and bicarbonate transport by cortical collecting ducts. A low urinary pH occurs during acidosis. It is caused by transport in the collecting ducts which are distal nephron segments. Regulation of acid-base transport in the distal nephron is believed to depend on the state of renal salt retention and the type and concentration of anion in tubular fluid. In order to investigate these interrelations the effects on cortical collecting ducts of desoxycorticosterone and of sulfate (an anion that facilitates urinary acidification) were investigated. Schwartz and Burg, using a microelectrode developed in this laboratory, measured the pH of fluid collected from isolated perfused cortical collecting ducts. Tubules dissected from desoxycorticosterone-treated rabbits did not acidify any more than tubules from normal animals. Therefore, no basis for the relation between sodium retention and acidification was found. Boyer and Burg investigated the effect of sulfate per-

fusion on bicarbonate transport, (which is an important component of urinary acidification). Previously, it was found that collecting ducts either secreted or absorbed bicarbonate depending on the acid-base status of the animals from which they were obtained. In the present studies sulfate stimulated bicarbonate absorption and/or inhibited its secretion. Further experiments are designed to determine the mechanism, and the specificity of the sulfate effect.

Tissue culture of kidney and other urinary epithelial cells

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 at those levels require much larger amounts of homogeneous tissue than are present in single tubules. Dr. Handler and his colleagues have been developing techniques for tissue culture of epithelial cells to overcome this difficulty.

In collaboration with Dr. Steele (Laboratory of Technical Development) a method has been developed for growing oriented epithelia on a filter and measuring voltage and short-circuit current under sterile conditions, following which the cells can be returned to culture. Ion fluxes can be measured simultaneously in order to identify the active transport processes. These methods serve both to screen different cell lines for transport processes and to characterize the transport. A number of existing epithelial cell lines have been screened in this way. The most promising is one derived from toad kidney. Its characteristics resembled those of collecting ducts. Transepithelial resistance was high, and transport (assessed by short-circuit current) was stimulated by aldosterone. There was evidence of high affinity binding sites for aldosterone, of metabolism of aldosterone to a more polar product, and of aldosterone-stimulated incorporation of amino acids into specific proteins.

Handler, Burg, Green and Perkins have been attempting to establish new kidney cell lines that demonstrate specific transport systems. The starting material has been single tubule segments dissected from rabbit or toad kidneys. The cells have been grown in culture for as long as four months (an estimated 10 to 15 generations). As yet there is not enough material to examine in detail any of the transport properties of the cells.

Handler and his colleagues have been studying lines of cells that they established from toad urinary bladders. The cells formed an epithelium that transported sodium actively from the apical to the basal surface. Two lines have been examined in detail. Cyclic AMP caused an increase in the rate of sodium transport and a fall in resistance. Although aldosterone stimulated sodium transport in both lines, resistance fell in one

line, as in the intact bladder, but not in the other line. The two lines also differed in their response to the transport inhibitor amiloride. These differences between lines of the same epithelial cells should be of value in understanding transport and its control, analagous to the use of mutants of bacteria. Although the cultured toad bladder cells responded to cyclic AMP, vasopressin did not stimulate their adenylate cyclase, as it does in intact toad bladders. Possibly, vasopressin receptors are lacking in the cultured cells or the receptors are not effectively linked to the cyclase. In order to test these possibilities Dr. Roy has prepared radioactive vasopressin to look for specific vasopressin receptors.

Once renal transport systems have been identified and characterized in tissue culture it should be possible to fractionate the cultured cells and isolate the transporters. As a first step functional membrane vesicles have been successfully prepared from the cells in tissue culture.

Epithelium of Necturus proximal tubule and gall bladder

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, the very large cells in the epithelia of certain amphibia, such as Necturus, are being used for direct measurements that elucidate the principles of epithelial cell transport.

1. NaCl transport by renal proximal tubules of Necturus. Spring and Shindo have continued to study the pathways and mechanisms of ion movement across proximal tubular epithelium. Previous experiments indicated that the entry of chloride into the cells from the lumen was dependent on the presence of sodium in the tubule fluid and was electrically neutral, implying that the cellular uptake of chloride was linked to sodium. Spring and Shindo are now investigating the exit of chloride from the cell across the peritubular border to see if it is by simple diffusion or is also a mediated process. The method is to clamp the voltage across the peritubular cell membrane by passing electric current through the epithelium from an axial metal electrode in the lumen. A double barrel intracellular microelectrode is used to monitor cell chloride activity and voltage. If there were simple diffusion of chloride across the peritubular cell border, the cellular chloride level should change with the cell voltage. No such change in cell chloride has been observed, however, implying that the chloride exit step at the peritubular border is also electrically neutral, linked to transport of another ion. The other ion that is involved remains to be identified.

2. Fluid transport and the dimensions of the cells and lateral intercellular spaces of Necturus gallbladder. Spring and Hope developed a system for clearly visualizing the cells and intercellular spaces of a living epithelium. Their method correlates structural changes with alterations in tissue function. By the use of video-computer methods cell shape and volume are measured rapidly and accurately in different experimental conditions. They observed that the cells shrank when NaCl was replaced by sucrose in the mucosal solution. The rate and amount of cell shrinkage provided a measure of the intracellular NaCl transport pool and active transport rate. They were also able to estimate the NaCl permeability of the apical cell membrane, and relative ionic permeabilities of the tight junction and lateral intercellular space. Experiments are presently under way to determine the other important cell membrane parameters, namely hydraulic water permeability, compliance, and solute reflection coefficients. Determination of these parameters is requiste for an exact understanding of the mechanism by which an epithelium absorbs salt and water.

3. Fluid transport and the osmolality of the lateral intercellular spaces. Spring and Leader worked to develop a method for optically measuring the concentration of solute in the fluid filling the spaces between gallbaldder cells. The fluid is thought to be more concentrated than the bulk solutions bathing the gallbladder. This point is of theoretical importance for analyzing the mechanism of fluid absorption, but there has been no way to measure the osmolality directly in order to validate the theory. Observation of the tissue with a microscope interferometer enables a measurement of the refractive index of the fluid within the spaces. (The refractive index is proportional to the concentration and osmolality.) Preparation of the tissue, measurement of standards, and operation of the microscope have all been completed and experiments are now being done with the tissue in different functional states.

Mechanism of hormone action in the kidney

Strewler and Orlcff investigated the mechanism by which vasopressin and its second messenger, cyclic AMP, affect the osmotic water permeability of renal tubules. They previously found that in a strain of mice with hereditary vasopressin-resistant diabetes insipidus there is a specific defect in protein phosphorylation in the renal medulla. Less ^{32}P appeared in phosphoproteins of molecular weight 175,000 and 185,000 in the affected animals than in normal controls. During the past year they confirmed this important result and found that the phosphoprotein was not present in renal cortex, liver, or brain. In older and younger mice with lesser degrees of diabetes insipidus, capacity to phosphorylate the specific phosphoproteins correlated with the urinary concentrating ability. Additional studies are required to test further whether the phosphoproteins are directly

involved in the action of antidiuretic hormone. If so, their isolation and characterization could provide important insights into the mechanism of hormone action.

Control of cell volume

Red cells of ducks and Amphiuma regulate their volume by the uptake or loss of ions and water in response to induced changes in cell volume and also in response to catecholamines. During the past year Siebens and Kregenow further characterized the system in Amphiuma red cells. During volume regulation, sodium chloride and water enter the cells, causing them to swell. The sodium uptake in Amphiuma (but not in duck) red cells was inhibited by amiloride, a drug previously best known as an inhibitor of sodium transport in epithelia. The action of the drug on the red cells resembled the action on epithelia with respect to kinetic characteristics, specificity, and reversibility. The Amphiuma red cells are large enough to be impaled by microelectrodes for direct measurement of voltage and resistance, using a method developed in this laboratory. The studies of volume regulation and amiloride action will be continued using this method. Kregenow has now found that chloride is important for volume regulation in duck erythrocytes. Although it was previously recognized that anions had to accompany the net movements of sodium and potassium during volume changes, the anions were presumed to move through independent transport systems or through leaks. Experiments in the past year revealed, however, that the sodium, potassium, and chloride transport are much more intimately linked, possibly by mutual transport on the same carrier. Thus, the increment in sodium and potassium permeation that results in volume regulation depends on the presence of chloride, and the increment in chloride permeation depends on the presence of sodium and potassium. The latter relation became evident when the normally high chloride exchange permeability of the cells was specifically inhibited with a disulfonic stilbene derivative. The stoichiometry of the sodium-potassium-chloride transport is now being studied.

Reconstitution of the red blood cell anion transport system

Cabantchik had previously identified "Band 3" protein to be the rapid anion exchanger in red blood cell membrane. He has now reconstituted the purified protein from detergent solution into liposomes and living cells. When reconstituted into liposomes, the Band 3 protein accelerated anion (sulfate) fluxes but not to the same extent as in native red cells. In order to test whether the difference was due to partial denaturation of the protein, it was reconstituted into Friend erythroleukemia cells (which lack the rapid anion exchanger), using Sendi virus membrane as a vehicle. Cabantchik showed that if the number of Band 3 protein molecules inserted into the Friend erythroleukemia cells is taken into account, there was qualitative and quantitative reconstitution of the transport system. Therefore, the quantitative

deficiency in liposomes is not due to denaturation of the protein. The method of reconstitution into the membranes of living cells should have general importance for assaying isolated transport components and assessing their physiological roles.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01216-04 KE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Pathways of salt movement across Necturus proximal tubules

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., LKEM, NHLBI

Other: Takahisa Shindo, M.D., Visiting Fellow, LKEM, NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Kidney & Electrolyte Metabolism

SECTION
Section on Electrolyte Transport

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 1/2	1 1/2	none

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The pathways and mechanisms of transepithelial transport of NaCl are being investigated in the proximal tubule of the Necturus kidney. Chloride and potassium sensitive intercellular microelectrodes were constructed to monitor intracellular chloride and potassium activity and to determine cellular responsiveness to change in the electrochemical gradient across the basolateral cell membrane.

667

PHS-6040
(Rev. 10-76)

Objectives

The pathways and mechanisms of salt absorption by renal proximal tubules are incompletely understood. Our first objective is the quantitation of the fluxes of Na^+ and Cl^- across both the cell and extracellular shunts of Necturus proximal tubules. We have previously shown that chloride ions enter into proximal tubule cells from the tubule lumen by a carrier mediated process. We wish to determine whether the exit of chloride from the cell to the blood is by simple diffusion or is a mediated process.

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.

An isolated perfused Necturus kidney is prepared so that complete control of the capillary composition may be achieved. Recordings of cell membrane potential and ion activities are made under control conditions. The tubule lumen is then perfused continuously with a test solution and the measurement repeated.

Major Findings

Steady state cell chloride and potassium activities were found to vary depending on the spontaneous electrical potential difference across the basolateral cell membrane. When the cell was more negative the intercellular chloride activity was lower and the potassium activity higher than the average, and vice versa. The variation in cell chloride with potential suggested that the basolateral membrane exhibits a significant chloride permeability.

Proposed Course

Continued study of intercellular ion activities in different functional states will permit analysis of cell membrane permeabilities and active transport kinetics. The relation between cell chloride activity and cell potential will be investigated.

Publications

1. Spring, K. R. and G. Kimura: Ion activities in Necturus proximal tubule. Fed. Proc. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 HL 01217-04 KE

PERIOD COVERED
October 1, 1978 to September 30, 1979

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., LKEM, NHLBI

Other: None

COOPERATING UNITS (if any)
Section on Theoretical Biophysics, NHBLI
Television Engineering Section/NIH

LAB/BRANCH
Laboratory of Kidney & Electrolyte Metabolism

SECTION
Section on Electrolyte Transport

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda,Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3/4	3/4	none

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

An optical-electrical system has been developed to enable precise, quantitative measurements of the dimensions of the cells and intercellular spaces of living flat epithelia. Light absorption due to spaces between cells is measured under varying physiologic conditions. The shape of the spaces as well as the relationship between the volume of the spaces and physical forces across the tissue are being investigated. Television image processing is used to accurately determine cell size and shape under different experimental conditions.

PHS-6040
(Rev. 10-76)

Objectives

The primary goal of this investigation is the generation of information about the role of changes in the geometry of the cells and intercellular spaces in the regulation of epithelial fluid transport. The methods employed represent a unique blend of optical and electrical techniques developed specifically for this investigation. We use on-line, real-time system for measuring the size and shape of the spaces and cells.

In addition to the data about the geometrical effects of physical forces on epithelial properties, information can be obtained on many of the physical properties of the tissue. Values for the ionic permeabilities and the hydraulic properties of the cell membranes should be obtainable.

Methods

The tissue of choice for these experiments is the gallbladder of the amphibian Necturus maculosus. The cells are 15 to 20 μM in diameter and 35 μM tall. The intercellular spaces are readily visualized and can be observed to open and close in response to changes in hydrostatic pressure or transport rate. This tissue is mounted in a specially designed chamber modified from a Dvorak-Stotler tissue culture chamber. The chamber is designed to allow the continuous perfusion of both surfaces of the tissue, measurements of tissue electrical properties, variation in transepithelial hydrostatic pressure, and rapid alteration of perfusate composition.

The chamber is placed on the stage of a Leitz inverted microscope equipped with brightfield optics. The viewing head is equipped with a beam splitter which is used to divert light from the image to a photomultiplier tube. The current output from the photomultiplier tube is used to indicate the size of the intercellular spaces. Adjustment of the slit of the photomultiplier enables measurements to be made from one intercellular space. The density of the light absorption by the space is proportional to the dimensions of the space thereby providing a quantitative measure of change in morphology. Position and focus of the microscope is monitored by observation of the preparation during measurements with the aid of a television camera. This camera is attached to the microscope beam splitter and contains an image intensifier so that low illumination levels may be achieved. 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 size and shape of the gallbladder cell and intercellular space were determined under control conditions. The intercellular space has a volume equal to 9% of the volume of the cell. The space volume may be varied by changing the hydrostatic pressure difference across the epithelium. The pressure-volume curves of the spaces were used to determine the compliance of the lateral cell membranes. The strength of active salt transport was measured by determination of the degree of dilatation of the intercellular spaces secondary to transport; changes in cell volume consequent to removal of mucosal NaCl were used to estimate the intracellular NaCl pool, active transport rate, and apical membrane NaCl permeability.

Proposed Course

We expect to obtain additional parameter values for the epithelium as a whole as well as for the lateral spaces. Measurements of the hydraulic water permeability and solute reflection coefficients of the cell membranes are planned.

Publications:

1. Spring, K. R. and Hope, A.: Fluid transport and the dimensions of cells, and interspaces of living Necturus gallbladder. J. Gen. Physiol. 73:287-305, 1979.

2. Spring, K. R. and Hope, A.: Dimensions of cells and lateral intercellular spaces in living Necturus gallbladder. Fed. Proc. 38:128-133, 1979.

3. Spring, K. R.: Optical techniques for the evaluation of epithelial transport process. Am. J. Physiol. Renal, Fluid and Electrolyte Physiology (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01219-03 KE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Tissue culture of epithelial cells from the urinary tract

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

P. I.: Joseph S. Handler, M.D., Head, Section on Membrane Metabolism LKEM, NHLBI

Other: M. B. Burg, M.D., Chief, LKEM, NHLBI
F. Perkins, M.D., Guest Worker, LKEM, NHLBI
C. Watlington, M.D., Guest Worker, LKEM, NHLBI
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A. S. Preston, Chemist, LKEM, NHLBI
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R. Kinne, M. D., Guest Worker, LKEM, NHLBI
Roderic E. Steele, Ph.D., TD, NHLBI

COOPERATING UNITS (if any)
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Dept. of Physiology, Yale Univ. Medical School
Dept. of Nephrology, WRAIR

LAB/BRANCH
Laboratory of Kidney & Electrolyte Metabolism

SECTION
Membrane Metabolism

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.8	4.8	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The program is intended to characterize existing lines of cells and develop new lines of cells that in culture perform transepithelial transport of interest to the nephrologist. We are attempting to start new lines of cells beginning with primary culture from microdissected segments of the nephron. Cells of lines cultured from the toad urinary bladder transport sodium actively. The rate of transport is stimulated by aldosterone and by cyclic AMP.

673

Objectives

The continuing purpose of this project is to develop techniques based on tissue culture that will afford better understanding of epithelial transport and its control by hormones and drugs. The need for this approach and its possible advantages have been discussed in previous reports. We have begun by trying to develop lines of cultured epithelial cells that manifest transport properties of interest to nephrologists. We are now examining these properties in detail.

Methods

See previous reports and annual report of R. Steele, Laboratory of Technical Development, NHLBI.

Major Findings

Primary cultures of epithelial cells from mammalian kidney have progressed to the point of growth of cells of epithelial morphology from dissected segments of the thick ascending limb and the cortical collecting duct of the rabbit. The cells have formed "domes," an indication of transepithelial transport by the cultured cells. The cells grow slowly. Some have been in culture as long as four months and are now on their third dish (about 10-15 generations). Cells of epithelial appearance have grown out of proximal and distal segments of the toad nephron. These cells are also on their third dish (10-15 generations at most). The cells derived from rabbit and from toad kidney have not grown to the point where they can be grown on filters and their transport properties assessed directly.

Cells derived from the toad urinary bladder have been in continuous culture for as long as two years. Five lines have been developed, two have been studied in detail. Line M has a transepithelial resistance (R) of 5300 ohm-cm^2 and short-circuit current ($I_{s.c.}$) of 8.6 $\mu A \cdot cm^{-2}$. In line 6c R is 9300 ohm cm^2, $I_{s.c.}$ is 2.2 $\mu A \cdot cm^{-2}$. Aldosterone stimulates $I_{s.c.}$, to 17.8 $\mu A \cdot cm^{-2}$ in line M and to 4.9 $\mu A \cdot cm^{-2}$ in line 6c. The stimulation of $I_{s.c.}$ in line M is accompanied by a fall in R, as occurs in the intact bladder. In contrast, the transepithelial resistance of line 6c does not change. The difference in these responses may indicate that aldosterone has more than one effect, one of which does not alter R. Cyclic AMP stimulates $I_{s.c.}$ in both lines. This is accompanied by a decrease in R in both lines. Amiloride is a diuretic that inhibits sodium transport by the distal nephron and toad urinary bladder, resulting in increased sodium excretion in the urine. In cells of line M, amiloride inhibits $I_{s.c.}$ under control conditions and after aldosterone. The cells of line 6c have a different response. Amiloride does not inhibit control $I_{s.c.}$ and after stimulation by aldosterone, amiloride

reduces $I_{s.c.}$ down to levels similar to controls. In line M, $I_{s.c.}$ is the equivalent of net sodium transport, as demonstrated by simultaneous fluxes of radioactive ^{22}Na and ^{24}Na under short-circuited conditions. The relationship holds under control conditions, and after stimulation by aldosterone and by cyclic AMP. In line 6c net sodium transport accounts for about 70% of $I_{s.c.}$. The nature of the small difference between net transport and $I_{s.c.}$, which persists after stimulation by aldosterone and by cyclic AMP, and after inhibition by amiloride, has not been determined.

Although vasopressin stimulates adenylate cyclase activity of the epithelial cells of the toad urinary bladder, it has no effect on the enzyme of the cultured cells.

In preliminary studies it was found that membrane vesicles could be prepared from the cells. The uptake of radioactive sodium by the vesicles displayed saturation kinetics and was inhibited by amiloride.

Significance

The continuous cell lines derived from toad urinary bladder have retained many transport properties and responses to hormones of interest. The cells should be of considerable value in gaining an understanding of these functions of the kidney. The response of line 6c to aldosterone and to amiloride is unlike that of line M and the intact toad urinary bladder. These differences in particular may lead to new understanding of the action of aldosterone and amiloride. If the kidney derived lines continue to develop, we should be able to use these techniques to study other transport functions of the kidney.

Proposed Course

Efforts will be continued to grow cells derived from kidney. The other lines of cells derived from toad urinary bladder will be characterized. The urea permeability and water permeability responses of the cells to cyclic AMP will be evaluated. In view of the fact that the toad urinary bladder cells have lost their adenylate cyclase responsiveness to vasopressin, we will examine the cells with radiolabelled vasopressin to see whether the cells have lost receptors for the hormone, or whether the defect is elsewhere.

Publications

1. Handler, J. S., Steele, R. E., Sahib, M. K., Wade, J. B., Preston, A. S., Lawson, N. L., and Johnson, J. J. Toad urinary bladder epithelial cells in culture. Maintenance of epithelial structure, sodium transport, and response to hormones. Proceedings of Natl. Acad. Sci, U.S.A.(in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01222-03 KE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Mechanism and control of calcium transport by the cortical thick ascending limb of Henle's loop

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

P. I. James E. Bourdeau, Ph.D., M.D., Research Associate, LKEM, NHLBI

Other; Maurice B. Burg, Chief, LKEM, NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Kidney & Electrolyte Metabolism

SECTION
Renal Mechanisms

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.8	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)

Calcium transport across the cortical thick ascending limb of Henle's loop has been studied in isolated perfused segments of rabbit renal tubules in vitro. Radioisotopic flux studies demonstrate passive calcium transport driven by the transepithelial voltage. Parathyroid hormone increases calcium absorption by stimulation of adenylate cyclase. Although parathyroid hormone rectifies the passive, voltage-dependent calcium transport in the absorptive direction under the conditions of our experiments, the mechanism by which the rectification occurs is uncertain.

PHS-6040
(Rev. 10-76)

Objectives

We previously found that calcium transport across the cortical thick ascending limb of Henle's loop perfused in vitro was strongly voltage-dependent and that the passive permeability of the thick ascending limb to calcium was approximately 25% of the sodium permeability. We also found an effect of parathyroid hormone to increase unidirectional lumen-to-bath calcium flux under conditions of high lumen positive voltage. This latter effect was mimicked by analogues of cyclic AMP (cAMP), but not by high concentrations of ACTH.

The purposes of the present study were further to examine the voltage dependence of calcium transport to test for active calcium transport in the presence of parathyorid hormone.

Methods

We perfused segments of single dissected cortical thick ascending limbs from rabbit kidneys in vitro. This technique was developed previously in this laboratory and enables us to measure simultaneously transepithelial voltage and radioisotopic (45Ca) calcium fluxes

Major Findings

1. When transepithelial voltage was reduced close to zero millivolts by perfusing and bathing the tubules with chloride-free solutions (nitrate substituted for chloride), the unidirectional lumen-to-bath and bath-to-lumen calcium fluxes were small, were not measurably different from one another, and were unaffected by parathyroid hormone. Since there was no net calcium transport near zero millivolts there was no evidence for active calcium transport with or without parathyroid hormone.

2. We tested whether lumen bicarbonate increases calcium transport across the cortical thick ascending limb, as previously suggested by others. We found that the presence of absence of bicarbonate in the lumen did not affect either the calcium fluxes or the transepithelial voltage.

3. Previously, we found that parathyroid hormone increased unidirectional lumen-to-bath calcium flux when voltage was highly positive in the lumen, that analogues of cAMP mimicked this effect of parathyroid hormone, and that ACTH had no effect on unidirectional lumen-to-bath calcium flux. In the present experiments we found that the reverse flux (bath-to-lumen) was very low and is not affected by parathyroid hormone under the same conditions. Combining these results net calcium absorption increases five-fold after addition of parathyroid hormone when transepithelial voltage is highly lumen-positive (the condition presumed to be present in the cortical thick ascending limb in vivo).

4. We tested the effect of parathyroid hormone on calcium transport at reduced lumen-positive and strongly lumen-negative voltages to test for a possible effect of the hormone on active calcium transport or the simple passive permeability of the epithelium to calcium. To control the voltages we used the "chemical voltage clamp" described previously from this laboratory. Our results at lumen positive voltages (+3.6 mV and +23.2 mV) are consistent with the hypothesis that parathyorid hormone acts to increase the passive transepithelial permeability to calcium. Net calcium absorption in the controls was related directly to voltage, and, after addition of the hormone, net calcium absorption increased in proportion to the voltage. In contrast, at negative voltages net calcium secretion decreased after addition of parathyroid hormone rather than increasing, as would be expected for an increase in simple calcium permeability. Taken together our results at all voltages demonstrate that parathyorid hormone rectifies net calcium transport in the absorptive direction, but does not stimulate active calcium transport. The question remains, however, whether there exists a specific membrane rectifier for calcium transport induced by parathyroid hormone or whether our results are merely a fortuitous combination of the effects of dilution potentials, nitrate substitution for chloride,and furosemide which we used to clamp the voltage.

5. Calcium permeability after addition of parathyroid hormone was calculated from the relationship between net calcium flux and voltage over the range of positive voltages (+1.3 mV, +3.6 mV, and +23.2 mV) and was found to be approximately equal to the sodium permeability previously determined in this segment.

Significance

Renal handling of calcium is important for its homeostasis. The thick ascending limb is an important site of calcium absorption. Elucidation of the voltage-dependent passive transport mechanism for calcium and its control by parathyroid hormone have provided important insights into the physiology and pathophysiology of its renal handling.

Proposed Course

This project has been completed.

Publications

Bourdeau, J. E. and Burg, M. D.: Voltage dependence of calcium transport in the thick ascending limb of Henle's loop. Am. J. Physiol. 236:F357-F364, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01224-03 KE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Control of sodium and potassium transport by isolated rabbit tubules

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

P. E.: Mark Knepper, M.D., Research Associate, LKEM, NHLBI

Other: Maurice B. Burg, M.D., Chief, LKEM, NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Kidney & Electrolyte Metabolism

SECTION
Electrolyte Transport

INSTITUTE AND LOCATION
NHLBI, NIH, 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)

This project is designed to delineate sites and mechanisms of hormonal control of renal sodium transport. Initially, proximal straight tubules (PSTs) from rabbits have been studied using the technique of isolated renal tubule perfusion developed in this laboratory. Early PSTs from superficial nephrons of rabbits treated chronically with deoxycorticosterone acetate (DOCA) prior to sacrifice had a significantly higher rate of iosotnic fluid reabsorption than the same segment from untreated rabbits. In addition photomicrographs reveal that the cells are thicker and the outer tubule diameters are greater in the DOCA-treated animals.

679

Objectives

Previous studies in cortical collecting ducts of rabbits have demonstrated that sodium reabsorption is stimulated by adrenal corticosteroid hormones. The effect of corticosteroids on sodium transport in other segments has not been clearly defined, however. The primary objective of this project is to determine the sites and mechanisms of corticosteroid control of renal tubular sodium transport.

Methods

We measured isotonic fluid reabsorption (J_v) in proximal straight tubules (PSTs) dissected from rabbits which have undergone various treatments. Treatment groups studied thus far include: 1) Control; 2) normal diet with chronic deoxycorticosterone acetate (DOCA) administration (5 mg/day IM for 7 to 11 days); 3) adrenalectomy; 4) sham adrenalectomy.

Major Findings

J_v in outer cortical PSTs (0.48±0.08 (SEM) nl mm·min) was significantly greater than in medullary PSTs (0.33±0.05 nl/mm-min) from superficial nephrons. All subsequent experiments were done exclusively in 1-2mm segments of PST dissected from the outer half of the renal cortex.

The mean J_v in PSTs from animals after chronic DOCA administration was significantly higher than in PSTs from untreated animals (1.06±0.04 vs 0.78±0.08 nl/mm·min, $p<0.01$). Photomicrographs of the perfused tubules revealed that the epithelial cells were thicker and the tubule outer diameters were uniformly greater in PSTs from DOCA-treated rabbits. These results could be due to a direct mineralocorticoid or glucocorticoid effect of DOCA on tubular transport or may be an indirect effect of DOCA, such as that resulting from changes in glomerular filtration rate (GFR) or extracellular fluid (ECF) volume.

There was no statistically significant difference in J_v in PSTs derived from adrenalectomized (0.62±0.0 nl/mm·min) rabbits. There was no consistent response in tubules from either adrenalectomized or sham-operated rabbits to the addition of 10^{-7} M aldosterone to the bathing fluid.

Significance

Although much has been learned in recent years about mechanisms of renal tubular epithelial transport of sodium, little is known about how the organism alters sodium transport

in response to changes in salt and water balance. Aside from the obvious importance to basic renal physiology, knowledge of such control mechanisms could add measurably to the understanding of the pathophysiology of various human disease states.

Proposed Course

Further studies in isolated rabbit PSTs will be carried out to investigate the means by which chronic DOCA administration increases J_v and causes tubular hypertrophy. Experiments will also be carried out to determine whether corticosteroids effect active chloride transport in isolated rabbit thick ascending limbs. Transmission electron microscopy will be done to determine what changes in cellular morphology occur in response to corticosteroid administration.

Publications

1. Warnock, D. G., Patlak, C. S., and Burg, M. B.: Contributions of leaked load to solute transport by renal tubules. Am. J. Physiol. 234:F480-F484, 1978.

2. Burg, Maurice B.: The nephron in transport sodium, amino acids, and glucose. Hospital Practice, October 1978, pp. 99-109.

3. Burg, Maurice B. and Stephenson, John L.: Transport characteristics of the loop of Henle. In" Physiology of Membrane Disorders, Thomas E. Andreoli, Joseph F. Hoffman, and Darrell D. Fanestil, eds., Plenum Medical Book Co., New York, 1978, Chapt. 33, pp. 661-679.

4. Schwartz, George J. and Burg, Maurice B. Burg: Mineralocorticoid effects on cation transport by cortical collecting tubules in vitro. Am. J. Physiol. 235:F576-F585, 1978.

5. Burg, M. B.:Chloride transport in the renal tubule. Fed. Proc. (in press).

6. Czaczkes, W., Vurek, U., and Burg, M.: Assay of sodium & potassium activated adenosine triphosphatase in submicrogram fragments of renal tubules. Analytical Letters (in press).

7. Burg, M. B.: The renal handling of sodium chloride, water, amino acids, and glucose, In: The Kidney, Barry M. Brenner, ed. 2d edition (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01225-03 KE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Control of cyclic nucleotide phosphodiesterase

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

P. I.: Gordon J. Strewler, M.D., Sr. Staff Fellow, LKEM, NHLBI

Other: Jack Orloff, M.D., Scientific Dir., IR, NHLBI
Vincent C. Manganiello, Ph.D., M.D., Med. Off.(Research) LCM, NHLBI
Martha Vaughan, M.D., Chief, LCM, NHLBI

COOPERATING UNITS (if any)
Laboratory of Cellular Metabolism

LAB/BRANCH
Laboratory of Kidney & Electrolyte Metabolism

SECTION
Electrolyte Transport

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

We previously demonstrated that cyclic nucleotide phosphodiesterase can be activated by a thiol protease which is released from a dense lysosomal fraction of rat kidney cortex by extraction with hypotonic media. A second more buoyant population of lysosomes appears to be deficient in protease activity. After purification to apparent homogeniety the protease exhibits a molecular weight of 23,000 and multiple charge isozymes (pI~5.6-6.0). Its substrate specificities and inhibitor sensitivities resemble those of cathepsin L from rat liver. Studies utilizing protease inhibitors provide no evidence that proteases participate in the hormonal regulation of phosphodiesterase in the intact cell.

682

Objectives

As part of our effort to elucidate the mechanisms of hormonal regulation of cyclic nucleotide phosphodiesterase (E.C. 3.1.4.17), we previously identified a thiol protease in rat renal cortex which activates phosphodiesterase. The objectives of the present study were to complete the purification and characterization of the protease, and to ascertain whether it has a role in mediating hormonal effects on phosphodiesterase.

Methods

A cyclic GMP phosphodiesterase from rat liver served as a substrate for phosphodiesterase activation; hemoglobin as a protease substrate; and benzyloxycarbamyl lysine p-nitrophenyl ester (Z-lys-pNP) and benzoyl arginine napthylamide (BANA) as substrates for esterase and amidase activity, respectively.

Major Findings

1. Phosphodiesterase activator has been purified 1200 fold to apparent homogeneity by hypotonic extraction of the particulate fraction of rat renal cortex, followed by sequential chromatography on DEAE cellulose, CM cellulose (pH 5), Sephadex G-75, and CM cellulose (pH 6.2). The purified enzyme is a thiol protease with a molecular weight 0f 23,000 by gel electrophoresis in SDS, and multiple charge isozymes (pH~5.6-6.0) on isoelectric focusing. It is capable of hydrolysis of the ester substrate Z-lys-pNP but not the amide substrate BANA. A BANA hydrolase which contaminated earlier preparations was separated from the protease by the last chromatography step. The substrate specificities and inhibitor sensitivities of the purified enzyme resemble those of cathepsin L from rat liver, and are distinguishable from those of cathepsin B, which exhibits BANA hydrolase activity. In addition, cathepsin B purified from porcine liver does not activate phosphodiesterase.

2. When the 600xg supernatant from rat renal cortex is centrifuged on a density gradient of colloidal silica (Cell, in press), phosphodiesterase activator, protease and BANA hydrolase activities sediment in a dense fraction containing several lysosomal hydrolase activities. A more buoyant fraction contains lysosomal hydrolase activities but no protease activity. This observation is consistent with the possibility of functional differentiation of kidney lysosomes.

3. The protease inhibitors leupeptin and antipain, known to penetrate into intact cells, were used to test the hypothesis

that a protease similar to the one we have identified mediates hormonal effects on phosphodiesterase in the intact cell. Leupeptin and antipain failed to block the effects of ACTH and insulin on phosphodiesterase in the rat adipocyte, and those of dexamethasone and dibutyryl cyclic AMP and theophylline on phosphodiesterase in HTC hepatoma cells. Thus we did not find evidence that a protease similar to the one we have studied is important in the control of phosphodiesterase activity in the intact cell.

Significance

1. We have isolated, characterized and determined the subcellular location of a protease whose properties suggest that it may play a major role in protein degradation by the kidney.

2. Our studies of the subcellular location of kidney proteases suggest that two populations of lysosomes in kidneys may be functionally distinct, in that one exhibits several protease activities and the other does not.

Proposed course: Termination.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01230-02 KE

PERIOD COVERED
October 1, 1979 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Control of water permeability

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

P. I.: Gordon J. Strewler, M.D., Sr. Staff Fellow, LKEM,NHLBI

Other: Jack Orloff, M.D. Scientific Director, IR, NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Kidney & Electrolyte Metabolism

SECTION
Electrolyte Transport Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	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)

It was previously shown that in a strain of mice with hereditary vasopressin-resistant diabetes insipidus there is a specific defect in protein phosphorylation in renal medulla. Two specific phosphoproteins, of molecular weights 175,000 and 185,000, display markedly diminished phosphorylation in homogenates from severely affected mice. These phosphoproteins are present in the particulate fraction of renal medulla from several murine strains with normal concentrating ability, and are relatively specific for this tissue, being undetectable in renal cortex, liver or brain. Additional studies have utilized young mice of the DI strain and mildly affected older mice. In both the urinary concentrating ability is intermediate between that of normal and severely affected mice, and both display an intermediate capacity to phosphorylate the proteins of interest. This finding suggests that the defect in phosphorylation is not simply coinherited with nephrogenic diabetes insipidus, but is directly related to urinary concentrating ability.

685

Objectives

It is established that cyclic AMP mediates the effect of vasopressin to increase the water permeability of mammalian collecting duct and amphibian urinary bladder, thus permitting concentration of the urine. By analogy with other systems, it has been proposed that the nucleotide does so by stimulating the phosphorylation or dephosphorylation of specific proteins. However the proteins involved have not been identified with certainty. We have examined protein phosphorylation in a strain of mice with hereditary vasopressin-resistant diabetes insipidus (DI mice). Since it appears, based on physiological studies of affected mice, that a specific defect in the cellular action of vasopressin results in decreased urinary concentrating ability, a comparison of protein phosphorylation in vasopressin-sensitive tissues of DI and normal mice could identify proteins whose phosphorylation is directly related to the control of water permeability.

Methods

The mice used included normal strain (VII +/+), a strain bred for a mild concentrating defect (DI +/+ non-severe) and mice bred for a severe defect (DI +/+ severe). Two groups of the latter strain were studied - young mice, who had not yet developed severe diabetes insipidus, and older animals, whose defect was severe. Proteins were phosphorylated by incubation of fractions with ^{32}P ATP and separated by gel electrophoresis.

Findings

1. 175,000 and 185,000 molecular weight phosphoproteins from normal renal medulla, whose phosphorylation was reduced in DI mice, were absent in liver and brain, from normal mice, implying a degree of specificity for vasopressin-sensitive tissues. They were also absent from renal cortex, perhaps because the vasopressin sensitive portion of the nephron makes up only a small portion of renal cortex.

2. In mice of the DI +/+ non-severe strain and young mice of the DI +/+ severe strain, whose ability to concentrate the urine was mildly reduced, there was more phosphorylation of the 175,000 and 185,000 molecular weight hands than in older mice of the DI +/+ severe strain, suggesting a relationship between phosphorylation of these proteins and the ability to concentrate the urine.

3. Extensive studies employing two-dimensional gel electrophoresis and sequential gel electrophoresis and isoelectric focusing failed to purify the proteins of interest sufficiently to allow quantitation of their phosphate content.

Significance

The observations presented are consistent with the view that phosphorylation of the 175,000 and 185,000 molecular weight proteins in renal medulla is directly related to urinary concentrating ability. Others have previously shown that vasopressin-sensitive adenylate cyclase activity is also reduced in renal medulla of DI mice. Further studies, perhaps employing isolated tubule techniques, will be required to determine whether either of these defects is causally related to vasopressin-resistant diabetes insipidus in the mouse.

Proposed Course: Termination

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01231-02 KE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Acid base regulation by cortical collecting ducts

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

P. I.: Jess Boyer, M.D., Guest Worker, LKEM, NHLBI

Other Maurice B. Burg, M.D., Chief, LKEM, NHLBI
George J. Schwartz, M.D., LKEM, NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Kidney & Electrolyte Metabolism

SECTION
Renal Mechenisms

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.3	0.2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The purpose of this project is to delineate the mechanism and control of acidification in the cortical collecting duct. Bicarbonate transport and pH are measured in isolated perfused collecting ducts dissected from rabbits. The role of various ions in modulating bicarbonate transport is being tested.

688

Objective

Recent studies from our laboratory showed that bicarbonate transport in cortical collecting ducts perfused in vitro was affected by the acid-base status of the animals from which they were dissected; i.e. tubules from acidotic animals absorbed bicarbonate and tubules from alkalotic animals secreted bicarbonate. The purpose of the present work is to investigate factors that modulate bicarbonate absorption and secretion and pH in the cortical collecting duct.

It has been suggested, based on indirect evidence, that the salt retaining ability of an animal, and the type and concentration of anion in tubular fluid affect acid-base excretion. We began by looking at these possible modulators of collecting duct bicarbonate transport.

Methods

Cortical collecting ducts are dissected and perfused as previously described. Total CO_2, predominately bicarbonate, is measured by the technique of microcalorimetry developed in this laboratory. A micro pH electrode, also developed in this laboratory, is used for measuring pH in collected tubular fluids. Some of the rabbits were treated with DOCA, 5mg/day for 7-21 days.

Major Findings

1. DOCA, a salt retaining hormone administered to the animals, had no measureable effect on the tubule fluid pH in vitro.

2. In acidotic rabbits the pH of tubular fluid collected at slow flow rates in vitro was higher than the urinary pH of the rabbits. At present it is not clear whether more distal segments (medullary collecting ducts) are specifically responsible for very acid urine pH in vivo or whether the cortical collecting ducts can acidify more, but are limited in vitro by a difference in conditions compared to in vivo. Both possibilities will be investigated.

3. Sulfate, an large anion present in normal urine, decreased bicarbonate secretion and increased bicarbonate absorption from cortical collecting ducts in vitro.

Significance

The collecting duct can make the final adjustments in urinary pH. Factors affecting proton and bicarbonate transport in both the cortical and medullary collecting ducts presumably are major determinants of urinary acid-base excretion. The

present studies indicate that sulfate, or anions like it, may modulate the process by inhibiting bicarbonate secretion or stimulating bicarbonate absorption in the collecting ducts.

Proposed Course

1. Further investigate the mechanism and specificity of the sulfate effect on bicarbonate transport.

2. Develop techniques for independently evaluating proton secretion (bicarbonate absorption) and bicarbonate secretion.

3. Investigate acidification in the medullary collecting ducts, and search for factors that might be affecting acidification in cortical collecting ducts in vivo different from in vitro.

Publications

1. McKinney, Thurman, D. and Burg, Maurice B.: Bicarbonate secretion by rabbit cortical collecting tubules in vitro. J. Clin. Invest. 61:1421-1427, 1978.

2. Burg, M. B. and Iino, Y.: Control of renal bicarbonate transport. Satellite Symposium of Epithelial Transport Mechanism," Kyoto, Japan (1978) (in press).

3. Burg, M. B.: Secretion and reabsorption of bicarbonate in single renal tubules of the rabbit., N. Y.Acad Sci. (in press).

4. McKinney, T. D. and Burg, M. B.: Bicarbonate transport by rabbit cortical collecting tubules. Effect of acid and alkali loads in vivo on transport in vitro. J. Clin. Invest. 60:766-768, 1977.

5. McKinney, T. D. and Burg, M. B.: Bicarbonate absorption by rabbit cortical collecting tubules in vitro. Am. J. Physiol. 2:F307-F314, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01232-02 KE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
CO_2 kinetics in rabbit renal proximal convoluted tubules

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

P. I.: George J. Schwartz, M.D., Sr. Staff Fellow, LKEM, NHLBI

Other: Alan M. Weinstein, M. D., Staff Fellow, OD, NHLBI
Roderic E. Steele, Ph.D., TD, NHLBI
John L. Stephenson, M.D., OD, NHLBI
Maurice B. Burg, M.D., Chief, LKEM, NHLBI

COOPERATING UNITS (if any)
Technical Development; Office of the Director

LAB/BRANCH
Laboratory of Kidney & Electrolyte Metabolism

SECTION
Renal Mechanisms

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md.

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 CO_2 permeability of rabbit renal proximal convoluted tubules was studied using the in vitro microperfusion technique. The permeability is very high (12×10^{-5} cm^2), indicating that the epithelium is not an important barrier to CO_2 diffusion.

691

PHS-6040
(Rev. 10-76)

Objective

The purpose of these studies was to measure directly the CO_2 permeability of rabbit proximal convoluted tubule in order to determine whether there might normally be a CO_2 partial pressure difference across the epithelium.

Method

Proximal tubules were perfused in vitro at flow rates of 86 to 976 nl/min·mm. Collected fluid pH and total CO_2 were measured with a glass microelectrode and microcalorimetry, respectively, and compared with the perfusate values. Bath pH was measured with a micro pH electrode. Appropriate analytic controls were carried out to assure that CO_2 was not lost from collected fluid between the time of collection and analysis. CO_2 partial pressure in perfusate, collected fluid, and bath was calculated from pH and total CO_2. Calculations and numerical analysis were performed on a Wang 2200 desk top computer.

Major Findings

Cellular hydrogen ion secretion, ammonia secretion, buffer transport and CO_2 production were found not to be of sufficient magnitude to significantly affect the tubular fluid pH at the flow rates used in this study.

The flux of CO_2 was measured with mean CO_2 partial pressure differences across the epithelium ranging from -50 to 150 mm Hg (negative value indicated higher pressure in the bath). The flux was linearly related to the pressure difference, (58 tubules $r=.94$) and the slope corresponded to CO_2 permeability of $6X10^{-5}$ $cm^3/s \cdot cm$ tubule length. The analysis included calculation amount of transported CO_2 which reacted with the various buffers known to be present in the perfusate.

The effect of the external unstirred layer was evaluated in two ways, by increasing the rate of stirring in the bath, and by adding carbonic anhydrase (0.5 mg/ml) to the bath. Carbonic anhydrase catalyzes interconversion of buffers at the boundaries of the unstirred layer in effect providing an additional pathway for CO_2 flux through the layer via diffusion of the buffer pairs that are formed. Both maneuvers caused approximately a 40% increase in apparent CO_2 permeability. From these results we calculated an unstirred layer thickness of 35 μm.

Internal unstirred layers (i.e. radial concentration gradients in the lumen) were also evaluated in two ways, by calculation and by addition of carbonic anhydrase to the perfusate. By

calculation the partial pressure at the inner wall of the tubule is approximately 25% less than the mean partial pressure of the perfusate. Adding carbonic anhydrase to the perfusate (0.5 mg/ml) results in a 45% increase in apparent CO_2 permeability.

We attempted to ascertain whether CO_2 diffusion within the cells normally is facilitated by endogenous carbonic anhydrase catalyzed reactions similar to the effect of the enzyme in unstirred layers. The addition of 10^{-4}M acetazolamide to the bath resulted in a 45% decrease in apparent permeability, suggesting that this is the case.

Mean CO_2 permeability was $12X10^{-5}$ $cm^3/s \cdot cm$ tubule length, taking into account the unstirred layers. This corresponds to a diffusion coefficient for CO_2 in the tubule epithelium of $1.3X10^{-5}$ cm^2/s which is half that measured for CO_2 in water at 37°C.

When the tubules were perfused with a CO_2 and bicarbonate-free solution and bathed in a solution containing 25 mm bicarbonate and 40 mm Hg of CO_2, the collected fluid of pH was approximately 0.3 units more alkaline than expected from the amount of CO_2 that had entered. Correspondingly the bicarbonate concentration was higher by a factor of 10 than predicted from the known bicarbonate permeability. The discrepancy is explained by reversal of the normal Na^+/H^+ exchanges at the lumen membrane providing luminal OH^- to react with CO_2 to form the additional bicarbonate.

We calculate that if all of the bicarbonate reabsorption by rabbit proximal tubules involves hydrogen ion secretion and generation of CO_2 in the lumen, the measured CO_2 permeability is so high a transepithelial partial pressure of less than 4 mm Hg suffices to reabsorb the CO_2 by diffusion. Thus, there should not normally be any very large transepithelial CO_2 pressure difference.

Significance

The kidneys control acid-base balance by varying the excretion of acid and bicarbonate. Since the bicarbonate/CO_2 buffer system is quantitatively the most important one in this process, adequate description of CO_2 kinetics is necessary for any satisfactory analysis. In this study an important parameter of the system, the CO_2 permeability of proximal tubules, has been directly measured for the first time.

Proposed Course

The action of an Na^+/H^+ exchanger may be involved in the $CO_2/HCO_3^-/H^+$ kinetics, as discussed above. Direct evidence for this relation will be sought by testing the effect of removing sodium both in the presence and absence of CO_2/HCO_3^-.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01233-02 KE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Molecular mechanisms of anion transport in red blood cells

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

P. I. : Z. I. Cabantchik, Ph.D., Visiting Scientist, LKEM, NHLBI

Other: Moshe Barzilay, Ph.D., Visiting Fellow, LKEM, NHLBI

COOPERATING UNITS (if any)
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LAB/BRANCH
Laboratory of Kidney & Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6/12	3/12	3/12

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The anion transport system in human red blood cells is studied by means of isolation of carriers, reconstitution, kinetic analysis, and chemical probes in order to define its molecular mechanism.

695

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(Rev. 10-76)

Objectives

The continuing goal of this project is to elucidate the molecular mechanism by which human red blood cell membranes facilitate the diffusion of anions. The present line of work emphasizes the identification, chemical characterization as well as isolation and reconstitution of the critical membrane components. The ultimate goal is to provide functional isolated systems in which transport processes can be dissected out into molecular events with the aid of chemical probes as well as with the aid of physicochemical techniques such as fluoresence spectroscopy.

Methods

Identification and isolation of transport systems

The obvious approach for identifying functional components of transport in biological membranes is to affix markers highly selective for particular systems. This should serve as a basis for tracking the transport system and expedite the development of fractionation procedures needed for isolating the critical components. The rationale which we adopted for designing markers is based on the systematic alteration of the substrate structure that leads to high affinity probes, i.e. competitive inhibitors which display 3 to 4 orders of magnitude higher affinities than the natural substrate. These properties are combined with covalent binding capacity to provide the definitive surface anchoring so as to attain a firm tagging confined largely to the microenvironment of the inhibitory site.

Chemical characterization of transport sites

The highly specific affinity probes capable of either reversible or irreversible binding are also an asset for characterizing and modelling the chemical architecture of substrate binding sites. By systematic variation of chemical groups in the probes, it was possible to relate the molecular structure of the probes to their inhibitory power. By kinetic analysis of inhibition, it was possible to determine the site of action as well as the stoichiometry of binding of probes to functional sites. By using impermeant forms of probes it was possible to study various characteristics of inhibition and of transport such as: sidedness, symmetry and mobility of functional sites. Finally, by integrating the various types of information a model for anion transport was developed.

Isolation and reconstitution of the anion transporter

Three new methods for isolating functional membrane polypeptides and for reconstituting them into either model membranes or plasma membranes of living cells were recently developed. The first, negative purification, consisted of selective removal of extrinsic membrane polypeptides leaving membrane residues enriched in the highly intrinsic 100,000 dalton (band 3) polypeptides, the putative anion transporter. The second, solubilization and reconstitution, consisted of solubilization of the above band 3 residue with detergent and reconstitution with exogenous phospholipids to yield monolamellar vesicles (in vitro reconstitution). The third, consisted of reconstituting band 3 triton x-100 extract together with triton x-100 extract of Sendai virus envelops. These hybrid vesicles fused spontaneously with Friend erythroleukemia cells, whereby band 3 was implanted into their plasma membrane and conferred upon them a Cl^- transport ability.

Major Findings

a. The anion transport system of human red blood cells has been extensively characterized by us and others both in terms of its kinetic and chemical properties. The role played by various chemical probes has undoubtedly been the key for recent developments in the field. In the studies this year, the inhibitory effects of aromatic sulfonic acids on sulfate exchange were determined. Two series of compounds were tested: benzene sulfonic (BS) and 2,2'-disulfonic stilbene (DS) derivatives. As judged by kinetic criteria the compounds acted at the sulfate transport site. Potency of inhibition (ID_{50}) varied over 10^4 fold (2-50,000 μM). The degree of inhibition depended principally on two chemical characteristics of the substituents: 1) liphophilicity (for chloro derivatives of BS) and electron donor acceptor capacity (for nitro, azido and amino derivatives of BS and DS). Based on these results, we suggest that the microenvironment of substrate recognition sites of the anion transport system bear positive charges and possess functional groups with electron donor capacity, embedded in a hydrophobic area. These chemical properties may define the susceptibility of the anion transport system not only to specific probes such as DS and BS but also to diverse agents such as diuretics, anesthetics and sedatives.

b. DNDS (4,4'-dinitro-2,2'-stilbene-disulfonic acid), an effective, reversible, non-penetrating agent was shown to specifically and competitively inhibit sulfate exchange in human RBC(25°C, pH 7.4) at μM levels. Full inhibition was accomplished when $8.0\pm7\times10^5$ molecules were bound per cell (measured by the Easson-Stedman procedure). Using various analytical procedures which included linear regression plots (modified Dixon and Hunter-Downs) and non-linear regression plots (Levenberg-Marquard) it

was demonstrated that the inhibitory effect was exerted on sulfate transport sites (K_i=0.45±0.1 μM). DNDS effects were asymmetric: no inhibition occurred from the inner surface of resealed ghosts (up to .1 mM DNDS at pH 7.4, 25°C). Permeability of cells to DNDS was also asymmetric. DNDS did not penetrate into cells or resealed ghosts (over a 2 hr. period) at 25°C or 37°C. DNDS (0.1 mM) exited from resealed ghosts at 36°C (30 μM/hr) but not at 25°C (measured over a 2 hr period). The irreversible inhibitor DIDS blocked DNDS exit, as well as DNDS binding to cells. The above data suggest that the asymmetry of the anion transport system is inherent in the properties of the transport sites themselves.

c. Anion transport in red blood cells involves a specialized obligatory 1:1 transmembrane exchange. The functional membrane component is an abundant intrinsic transmembrane polypeptide of 95,000 daltons (band 3). Despite the symmetric nature of the transport process, there is a sidedness in the susceptibility of functional sites to inhibition by derivaties of highly specific probes, such as 2,2'-disulfonic stilbenes (DS) and 2-(aminophenyl) -6-methylbenzenethiazol-3,7-disulfonic acid (APMB). The non-penetrating probes 4-nitro-4'-amino-DS (NADS) coupled to dextrans (MW 10,000 or 40,000) (D) via amidoalkyl arms of variable length and 4,4'-dinitro-DS (DNDS) were applied either internally or externally to resealed ghosts. They inhibit only from the outer surface, irrespective of the arm length. The slowly penetrating probe APMB inhibited from either surface. Derivatives of APMB coupled to D inhibited from the outside, irrespective of the arm length, but only inhibited from inside when there was a long alkyl arm between APMD and D. Based on these results we suggest: 1) that the reason for the sidedness of inhibition is that the functional transport sites are located geographically closer to the outer membrane surface and 2) that the actual barrier over which transport occurs extend over a fraction (1/2 or less) of the anatomical width of the membrane.

d. In addition to two previous methods we developed for isolation and reconstitution of the functional components of anion transport (i.e. band 3 proteins) we have recently accomplished functional incorporation of the anion exchange into Friend erythroleukemia cells by fusing either red blood cells or isolated band 3 with Sendai virus. The Cl^- exchange capacity of the Friend cells was significantly stimulated. The stimulation was blocked by pretreating the red blood cells with DIDS which irreversibly inhibits the anion carrier. This method of functional incorporation of transport proteins into viable receptor cells that lack them provided a useful assay for reconstitution of transport components.

Publications

1. Cabantchik, Z. I., Knauf, P. A. and Rothstein, A. The anion transport system of the red blood cell. The role of membrane protein evaluated by the use of probes. Biochim. Biophys.. Acta Reviews in Biomembranes 515:1-64, 1978.

2. Barzilay, M., Ship, S. and Cabantchik, Z. I. Anion transport in red blood cells. I. Chemical properties of anion recognition sites as revealed by structure-activity relationship of aromatic sulfonic acids. Membrane Biochem. 2:227-254, 1979.

3. Barzilay, M. and Cabantchik, Z. I. Anion transport in red blood cells. II. Kinetics of reversible inhibition by nitroaromatic sulfonic acids. Membrane Biochem. 2:255-281, 1979.

4. Barizlay, M. and Cabantchik, Z. I. Anion transport in red blood cells. III. Site and sidedness of inhibition of high affinity probes. Membrane Biochem. (In press).

5. Cabantchik, Z. I., Volsky, D. J., Ginsburg, H. and Loyter, A. In vitro and in vivo reconstitution of the erythrocyte anion transport system. Ann. N. Y. Acad. Sci. (In press).

6. Volsky, D. J., Cabantchik, Z. I., Beigel, M. and Loyter, A. Implantation of the erythrocyte anion channel into plasma membrane of Friend erythroleukemia cells by Sendaii virus envelopes-mediated fusion. Sendai virus envelopes-mediated fusion. Proc. Natl. Acad. Sci. USA. (In press)

7. Beigel, M., Volsky, M., Ginsburg, H., Cabantchik, Z. I. and Loyter, A. Functional incorporation of the human erythrocyte chloride exchange system into plasma membrane of Friend erythroleukemia cell of Sendai virus induced cell fusion. (In press)

8. Cabantchik, Z. I. The isolation and reconstitution of the anion transport system of human red blood cells: in vitro and in vivo approaches. Alfred Benzon Symposium on Transport of Ions Across Erythrocytes. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01234-02 KE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Osmolality of the lateral intercellular spaces of a living epithelium

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., LKEM, NHLBI

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COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Kidney & Electrolyte Metabolism

SECTION
Section on Electrolyte Metabolism

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2/12	2/12	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The osmolality of the fluid within lateral intercellular spaces separating gallbladder epithelial cells is being determined from measurements of the refractive index. Living Necturus gallbladder epithelium is placed in a special chamber and observed with a microscope interferometer. The refractive index of the cells and the spaces between them are being compared to a surrounding cell-free area.

700

PHS-6040
(Rev. 10-76)

Objective

Fluid transport by epithelia is secondary to active solute transport into the lateral intercellular spaces separating epithelia cells. Several recent theories predict that the spaces are filled with a fluid similar in concentration to that in the bulk solutions bathing the tissue, while the earlier models indicated that the intercellular fluid is more concentrated than the bulk solution. It is the object of this project to measure the osmolality of the intercellular fluid by determination of its refractive index, in situ, to resolve the question.

Methods

Necturus gallbladder is mounted in a special perfusion chamber after removal of the serosal connective tissue. This chamber permits the continuous perfusion of both mucosal and serosal baths and ideal optical properties. The tissue is supported on a titanium electron microscopy grid and is observed at 1000x magnification on a Mach-Zehnder microscope interferometer. Video images of interference fringe patterns are analyzed illumination at several different wave lengths.

Major Findings

We have been able to measure the osmolality of test solutions within a few percent. Similar accuracy has been achieved on the living epithelium. Difficulties in interpretation of the tissue measurements have prevented meaningful calculations of interspace osmolality. Interspace fluid osmolality has been determined in transporting and non-transporting states, and the refractive index computed by two methods. The results with the two-wavelengths method (narrow fringes at two or more wavelengths of monochromatic blue-green light) cannot be completely corrected for interferences from the junctional complex and overlying cytoplasm.

Proposed Course

We will attempt to add a small amount of highly refractive solute to the solution in the lateral intercellular spaces to calculate the interference due to the junctional complex. We will improve the illumination and image processing components of the system. We may utilize epithelial cells from tissue culture because of their favorable geometry.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01236-02 KE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Volume regulation in Amphiuma red cells - Effect of amiloride

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
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	10/12	2/12

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

We have continued our studies of volume regulation in the giant red cells of Amphiuma in preparation for their use in micropuncture measurements. Amphiuma red cells regulate their volume in anisotonic media by utilizing transport mechanism(s) that selectively control the cells' ability to gain or lose electrolytes. The process whereby cells enlarge in hypertonic media involves an amiloride sensitive Na uptake. This finding has led to the suggestion that the Na transporter in Amphiuma erythrocytes and that present at the mucosal border of "tight " epithelia is similar. Additional evidence in support of this hypothesis has been presented.

PHS-6040
(Rev. 10-76)

Objectives

Red cells from several species have been shown to regulate their volume in anisotonic media. They accomplish this by using unique transport mechanisms, which allow the cell to selectively lose or gain electrolytes. Water accompanies the electrolyte and produces the adjustment in size. We began this study to determine whether the giant red cells of Amphiuma means are able to regulate their volume. These cells, because of their size, are large enough to be studied by micropuncture. If these cells are able to regulate their volume, it should be possible to study these transport systems via a single-cell micropuncture technique developed earlier by us. Last year we found that these cells do possess the necessary transport mechanisms. In addition, we showed that cells enlarge by selective uptake of Na which is inhibited by amiloride. Since Na uptake at the mucosal border of "tight" epithelia is also blocked by amiloride, we suggested that the Na transporter in the Amphiuma red cells and that at the mucosal epithelial border is similar. This year's study examines this hypothesis further and continues to assess the means whereby Amphiuma erythrocytes regulate their volume in anisotonic media.

Methods

The methods have been described in detail in the previous annual report.

Major Findings

Hypotonic response

We previously found that cells which have initially enlarged 30% in hypotonic media return in 7 hrs. to a volume that is approximately 10% larger than normal. The K loss results from 10-20 fold increase in K efflux.

1. We now find that during this response net chloride loss is about 1/3 the magnitude of the cation loss.

2. During the response Na uptake also increases and produces an increase in pump activity. The increased pump activity maintains the normal low Na levels of the cell. However, when the pump is blocked with ouabain this additional Na accumulation becomes apparent and is compensated for osmotically by an equivalent additional loss of K. Thus, during the response the sum of cellular Na and K content at any given time is the same in the presence and absence of ouabain.

Hypertonic response

Previous studies indicated that cells shrunken 20% in hypertonic media rapidly gain Na and enlarge to nearly their original volume in about an hour. In this response, Na influx increases by two orders of magnitude. Ouabain has no affect on the volume changes or the total amount of newly accumulated cation, although by inhibiting the pump, it does block a gradual exchange of the new Na for extracellular K. The diuretic, amiloride, inhibits the increase in Na influx, preventing enlargement. Fifty percent inhibition occurs at an amiloride concentration of 10^{-5} M. To provide support for our contention that the "Na transporter" is similar in both Amphiuma red cells and the mucosal border of "tight" epithelia, we examined further the response of Amphiuma red cell to hypertonicity. Additional features of the hypertonicity-induced transport process which have previously been observed in epithelia include:

1. The inhibition produced by amiloride is readily reversed by washing the erythrocytes in amiloride-free media.

2. The increase in Na influx is a saturable function of external Na. The curve describing Na influx and extracellular Na follows Michaelis-Menten kinetics and has a K_m of approximately 24 mM. This value is in fair aagreement with those reported for frog skin, toad bladder and trout gill.

3. Lithium can replace Na in the enlargement process while potassium cannot. Lithium competitively inhibits the stimulated Na influx.

Other characteristics of the response, some of which have been observed in epithelia, include the following:

1. Chloride accompanies the Na. In the initial 15 minutes, the net Cl uptake is about the same or slightly greater than the net Na gain. Later in the response, however, the gain in Na exceeds the increase in chloride so that at one hour the net Cl uptake is about 75% of the net Na uptake.

2. Upon placing the cells in hypertonic media, the Na influx accelerates gradually, reaching a maximum rate at 20-30 minutes.

3. Removing amiloride late in the response produces a sudden uptake of Na which is greater than the comparable rate had amiloride been absent. This enhanced Na uptake, because of its magnitude, may be useful in future studies in which we hope to determine whether Na enters the cell through an electrically silent or a conductive pathway.

4. Although the response to hypotonic media appears to be basically similar in the Amphiuma and the duck red cell, the response to hypertonicity differs. First, duck red cells in the presence of ouabain gain both K and Na. K plays the major role initially. In the Amphiuma red cell, only Na enters the cell in significant quantities initially. Second, duck cells require both Na and K in the medium for enlargement to take place, while Amphiuma cells are able to enlarge in the absence of external K. Third, amiloride is without effect in duck cells, whereas it completely inhibits enlargement in Amphiuma cells. Conversely, furosemide is without effect on Amphiuma red cells while it inhibits enlargement in duck red cells.

Significance

1. Amiloride acts on various epithelia by preventing the entry of Na into the cells, resulting in an inhibition of net Na movement across the cell. The characteristics typically used to establish that a common amiloride-sensitive Na transport mechanism exists in these epithelia are also shared by Amphiuma erythrocytes in their response to hypertonicity. Amphiuma red cells may prove useful in further characterizing and possibly isolating this Na transport system.

2. The demonstration that Amphiuma red cells are able to control their volume in anisotonic media is the first step in the direct study of volume regulation through micropuncture.

3. The differences between the responses to hypertonicity in Amphiuma red cells compared to those of the duck indicate that either Na or K may serve as the predominant ionic species in cell enlargement.

Proposed Course

1. Studies of volume regulation using the single cell technique have begun. We aim to describe the electrical characteristics of the responses to hypotonicity and hypertonicity, and hope to be able to use electrical parameters to help select among possible mechanisms for net salt movements during these responses.

2. The role of anions will be further explored, particularly the degree to which these transport mechansims are dependent on chloride.

Publications: In preparation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01237-01 KE

PERIOD COVERED
October 1, 1978 to September 30, 1979

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

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Other: J. S. Handler, M. D., Chief, Membrane Metabolism, LKEM, NHLBI
J. P. Johnson, Ph.D., Major, M.C., WRAIR
M. Matsumura, M.D., Visiting Fellow, LKEM, NHLBI
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COOPERATING UNITS (if any)
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SECTION
Membrane Metabolism

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md.

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)

Toad kidney cells grown in culture respond to 10^{-7} M aldosterone with an increase in short-circuit current. The binding of ^{3}H-aldosterone to whole cells yields results consistent with the presence of a high affinity receptor. A metabolite of aldosterone also appears to be present. Studies are planned to examine binding of aldosterone to receptors in the cytoplasm and in the nucleus as well as studies to identify a specific protein or proteins induced by aldosterone.

706

Objectives

There is limited understanding of the mechanism of action of hormones on transepithelial transport. Among the limitations or handicaps of previous studies of intact organs or tissues, is heterogeniety of the population of cells under study (e.g. rat kidney tubules, toad urinary bladder), and the heterogeneity and lack of control of the nutritional, endocrine, and genetic background of the material studied. A large portion of these difficulties might be overcome by examining the effect of hormones on transporting epithelia grown in culture.

In the past year we have identified a line of cells (A-6), derived from the kidney of the toad, that in culture demonstrates transepithelial transport properties and responds to aldosterone. It is the purpose of this study to examine transport by these cells in culture and the regulation of transport by aldosterone.

Methods

The cells s are maintained in culture in a mixture of Coon's modification of Ham's F-12 and Lebovitz' L-15 (modified to contain electrolytes appropriate for amphibia) with 10% fetal bovine serum. Transport is assessed by growing the cells on collagen coated nucleopore filters (see R. Steele, annual report, L.T.D., NHLBI) and measuring transepithelial electrical potential difference, short-circuit current ($I_{s.c.}$) and net ion and water flux. Aldosterone binding by receptors in cytosol and nucleus is examined by incubating the cells with different concentrations of ^{3}H-aldosterone, rinsing the cells free of medium containing aldosterone, and collecting the cells in the cold. Nuclear and cytosol fractions are separated by centrifugation. The labelled material eluted from cells is characterized by thin layer chromatography. The effect of aldosterone on the enzyme Na-K ATPase, (believed to be the sodium pump) in the basolateral plasma membrane of these cells, is assessed by measuring the activity of the enzyme in a standard assay and/or by measuring "specific binding" of ^{3}H-ouabain. Specificity of ^{3}H-ouabain binding will be judged by displacement of bound ^{3}H-ouabain by high concentrations of unlabelled ouabain and by high concentrations of K^{+}. Binding is assessed in intact cells and in broken cell preparations.

The effect of aldosterone on the incorporation of radiolabelled amino acids is studied in order to assess the induction of protein synthesis by the hormone. Cells on petri dishes are incubated with radiolabelled amino acids, controls with one isotopic label, aldosterone treated cells with another. The cells are rinsed free of label, collected by scraping, and pooled before they are homogenized, fractionated by centrifugation, and their proteins separated by SDS polyacrylamide gel electrophoresis.

Major Findings

The cultured toad kidney derived epithelial cells form a monolayer when grown on plastic petri dishes or on collagen coated nucleopore filters. The cells grown on petri dishes form domes, an indication of apical to basolateral fluid transport. When seeded on filters at densities of 4.0 to 9.0x10^5 cells/cm^2, a transepithelial electric potential difference (P.D.) develops within 24 to 48 hours, and reaches a stable peak value of 4.4±0.2 mV (apical surface negative) after 72 to 96 hours. Short-circuit current ($I_{s.c.}$) and transepithelial resistance follow a similar time course, with stable values of 1.1±0.1 μA·cm^{-2} and 4000Ω·cm^2.

The transport effect of aldosterone is assessed in cells grown in media containing charcoal treated serum (the charcoal strips the serum of lipids, including steroid hormones, and other factors). $I_{s.c.}$ is stimulated slightly by 10^{-9} M aldosterone and maximally (5.2±0.4 μA·cm^{-2}) by 10^{-7} M aldosterone.

Preliminary studies of the binding of ^{3}H-aldosterone to the cells grown on a petri dish are consistent with the existence of high affinity binding at 10^{-10} and 10^{-9} M aldosterone and a second binding component at 10^{-8} and 10^{-7} M aldosterone. The latter may represent binding to a glucocorticoid receptor. Thin layer chromatography of the ^{3}H-labelled material extracted from the cells incubated with ^{3}H-aldosterone, reveals two peaks, one corresponding to aldosterone, the second, more polar peak, a metabolite of aldosterone. At 28°C the amount of ^{3}H-aldosterone bound to cells is stable between one and three hours, the amount of metabolite continues to increase with time. At 4°C, ^{3}H-aldosternone binding is similar to that at 28°C and the amount of metabolite is markedly reduced. These conditions (4°c) should be suitable for initial studies of aldosterone binding to the cells. Adenylate cyclase activity of the toad kidney cells is stimulated by fluoride, guanine nucleotides, and by prostaglandin E. Vasopressin, parathyroid hormone, calcitonin, glucagon, and epinephrine do not stimulate the enzyme.

Na-K ATPase activity in the cells, as in toad urinary bladder epithelia, is a small (< 10%) fraction of total ATPase activity. ^{3}H-ouabain binds to intact cells and to the 30,000 x g pellet of a homogenate of cells, and appears to be the more reliable method for quantifying the number of Na transport sites.

In preliminary experiments, incubation with aldosterone for four hours and for six hours resulted in increased incorporation of methionine into proteins with molecular weights in SDS of 14,000 and 60,000. The proteins are located in a 12,000 x g supernatant and pellet respectively.

Significance

Aldosterone plays a major role in the regulation of salt metabolism. This preparation of cultured kidney cells should be of considerable value in gaining further understanding of the mechanism of action of aldosterone.

Proposed Course

The relationship between $I_{s.c.}$ and net sodium transport will be determined. The relationship between the aldosterone concentration and the $I_{s.c.}$ response will be compared to the specific binding of aldosterone under the same conditions. Binding of aldosterone to cytosolic and nuclear receptors will also be evaluated. The effect of aldosterone on amino acid incorporation will be confirmed and the location of the protein in broken cells determined. Finally, the effect, if any, of aldosterone on Na-K ATPase will be estimated.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01239-01 KE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
The role of chloride in the volume regulatory transport mechanisms

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

P. I.: Floyd M. Kregenow, M.D., Sr. Investigator, LKEM, NHLBI

Other: Theresa Caryk, Chemist, LKEM, NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Kidney & Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 1/2	9/12	9/12

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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)

Under physiological conditions, the transport mechanisms that regulate cell volume in duck erythrocytes require Cl. Cl seems to be co-transported with K or Na+K during these transport processes. This dependence on Cl is a linear function of the intracellular and/or extracellular chloride concentration. By inhibiting the chloride fluxes associated with the anion exchange mechanism, one can unmask these cation-coupled chloride fluxes and observe them directly. The evidence indicates that this Cl movement occurs through membrane pathways not related to the anion exchanger.

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Objectives

Transport processes which control cell size were first described in this laboratory using duck erythrocytes. Cells change their volume by using these transport systems to selectively lose or gain electrolytes. Shifts in cell water accompany the electrolyte changes and produce the size adjustments. Initial studies indicated that two separate pathways exist; which one operates depends upon whether the cells need to swell or shrink to return to their original size.

There were several reasons for believing that both systems were basically cation (Na or K) transporting mechanisms, although an anion (primarily chloride) also is needed to accompany the cation in its passage across the membrane. First, cation fluxes appeared to be rate limiting; Cl fluxes were a million times more rapid and were apparently not rate limiting. Most of this Cl movement occurs through an anion exchange pathway that obligatorily exchanges Cl for Cl or some other anion in a one to one fashion. Second, and most important, varying the Na or K electrochemical gradient produced predictable changes in the Na or K fluxes, Na and K net movement, and the volume changes. Finally, furosemide, an inhibitor of a cation transport system in human erythrocytes (Pump II) also inhibited the transport mechanisms by which cells enlarge.

An important unanswered question is how extensively these volume regulatory transport mechanisms are distributed in the animal kingdom. Although they have been described subsequently in other nucleated erythrocytes, as well as in several continuous tissue cell culture lines, their distribution remains unclear. Last year's annual report suggested that the transport process responsible for enlarging Amphiuma red cells was similar to that responsible for NaCl entry at the mucosal border of some epithelia. This finding prompted the suggestion that this transport system had been modified in at least some epithelia to serve as part of the epithelial electrolyte transporting apparatus. Because many epithelial transporting systems have been shown recently to depend uniquely upon Cl, we decided to reinvestigate our previous findings related to the anion dependence of these transport processes. In our earlier study we showed Br but not SO_4 could replace Cl as the anion which accompanies Na and K. We undertook this study then to explain the nature of the SO_4 inhibition.

Methods

The basic techniques and procedures have been described in previous annual reports. Thin layer chromatography of PAH (para aminohippuric acid) was performed on Silica G plates using a mixture of methanol, NH_4OH and water as the solvent system.

Major Findings

The self exchange of Cl, Br, I, NO_3, SCN, and SO_4 across the membrane of duck erythrocytes or the exchange of one of these inorganic anions for another is very rapid, as it is in human red cells. This rapid anion equilibration, which occurs through the anion exchange mechanism, prevents one from experimentally restricting any of these anions to only one side of the membrane. On the other hand, PAH, an organic anion is essentially non-permeating at physiological pH, and can be isolated at the inside or outside of the cell.

Both transport systems (i.e., the one responsible for shrinking and the one responsible for enlarging cells) operate when Br instead of Cl is present. Cation fluxes, though, are 5-10% slower with Br present.

Both transport systems become non-functional when I, NO_3, SCN, or SO_4 completely replace Cl. Thus, one no longer observes an increase in K efflux when cells are placed in hypotonic media (shrinkage), or an increase in K influx, K efflux, Na influx, or Na efflux when cells are stimulated with norepinephrine or incubated in hypertonic media (enlargement). Inhibition even occurs with HCO_3 present, which also crosses the membrane via the anion exchange mechanism.

If instead of complete replacement, one replaces only part of the chloride, then cation transport and volume regulation become a function of the remaining Cl. That is to say, cation transport decreases as the Cl decreases. This relationship between cation transport and Cl is linear. All 4 inorganic anions (I, NO_3, SCN, SO_4) act similarly provided one takes into account the small osmotic effect that occurs when SO_4 replaces Cl. The same relationship between Cl and cation transport also exists if chloride is replaced with the impermeant anion, PAH. Replacement was accomplished through a new technique which leaves cell volume and membrane potential $\frac{(Cl)_o}{(Cl)_i}$ undisturbed.

These findings indicate that SO_4, I, NO_3, SCN, and HCO_3 can not serve as the required anion necessary for cation transport to occur; whereas, Cl and Br can. The first 4 anions also seem to inhibit by simply replacing and reducing essential Cl. Thus, under physiological conditions, cation movement is linked to Cl movement in a way that depends upon a linear relationship with the chloride concentration. Finally, the membrane pathway followed by this chloride is different than that used by the anion exchanger.

Further evidence that this chloride follows a membrane pathway not related to the anion exchanger comes from experiments with Sits. Sits, a specific inhibitor of the anion exchanger, has no effect on the cation fluxes, net cation movements or

volume changes produced by either transport mechanism. In the presence of maximally effective concentrations of Sits, Cl fluxes associated with the anion exchanger are sufficiently reduced to unmask the cation coupled Cl fluxes. Thus, under these conditions, we can vary the extracellular Na and K concentrations and measure directly Cl fluxes that accompany those cation fluxes associated with the enlargement process. Coupled Cl fluxes even occur when the cation movements describe a process resembling exchange diffusion. There is therefore an exchange of KCl for KCl rather than K for K under these conditions. Direct Cl flux measurements associated with the process of cell shrinkage can also be obtained by comparing the Cl fluxes of enlarged cells containing only K or Na. Since only the K cells can shrink, the additional Cl efflux associated with these cells represents Cl leaving with K.

In the course of these experiments, we found that isotonic control avian erythroytes, like human erythrocytes (Pump II of Kregenow and Hoffman, 1966) have a component of Na influx, Na efflux, K influx, and K efflux that is ouabain insensitive. These flux components are responsive to variations in the extracellular Na or K concentration, and are inhibitable by the addition of furosemide or the removal of Cl. These same characteristics define the cation fluxes associated with the process whereby cells enlarge. During the enlargement process, however, the fluxes are orders of magnitude larger and lead to cell swelling under the proper conditions. The marked similarity with which these same agents or experiental manipulations affect cation transport in isotonic control avian or human erythrocytes suggest that a similar transport process operates in these cells. Transport in these isotonic control cells, though, is much reduced and the cation fluxes behave as if they were part of an exchange diffusion process.

Significance

1. The observations that led to the conclusion that Cl is essential to both transport processes and functions as if it were co-transported with K or Na significantly enhances our understanding of how these transport mechanisms operate. Importantly, these observations limit the possible ways transport could occur.

2. The parallelism with which Cl operates in both volume regulatory processes indicates additionally that both transport mechanisms may have basic similarities.

3. One of these transport mechanisms may function in a reduced and altered form in isotonic cells. Whether a cell is able to enlarge may then depend only upon its ability to amplify this

basic transport process. The duck erythrocyte has this ability; the human erythrocyte does not.

Proposed Course

We plan to obtain coupling ratios between the Na, K, and Cl fluxes.

Publications:

Stoner, Larry C. and F. M. Kregenow: A single cell technique for the measurement of membrane potential, membrane conductance and the efflux of rapidly penetrating solutes in Amphiuma red cells. (submitted to J. Gen. Physiol.)

Annual Report of the
Laboratory of Molecular Hematology
National Heart, Lung, and Blood Institute
October 1, 1978 to September 30, 1979

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 Unit of Molecular Genetics, which is primarily concerned with the molecular control of eukaryotic gene expression; the Unit of Molecular Cloning, which is primarily concerned with the isolation and characterization of globin genes from the genomes of eukaryotic cells; and the Unit of Protein Biosynthesis, which is primarily concerned with the mechanism and regulation of hemoglobin synthesis at the translational level.

UNIT OF MOLECULAR GENETICS

The immediate objectives of this Unit are to: (1) identify, isolate and characterize the regulatory factors controlling the expression of the globin genes, and (2) identify the regulatory regions of animal and human DNA which are involved in the control of the expression of the globin genes. 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 beta thalassemia and other diseases involving abnormal hemoglobin biosynthesis.

Regulatory factors controlling the expression of the globin genes are being sought by a combination of cell biology and molecular biology techniques. Somatic cell hybrids, 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 are being established.

The Unit has succeeded, during the past year, in demonstrating that:
(1) the positive regulatory factor(s) that is induced in 2S MEL-human fibroblast hybrid cells is globin gene specific. Namely, it is involved in the turning on of the human beta globin gene (as well as the mouse alpha and beta globin genes), but does not activate the closely linked human gamma globin gene. Its mechanism of action appears to be at the transcriptional level since gamma globin mRNA sequences cannot be detected in the nuclei of induced hybrid cells. In addition, induction of the positive regulatory factor(s) occurs with several different inducers. This is the first example of a gene specific regulatory factor in a eukaryote.

(2) The gamma-delta-beta globin gene complex is located on the short arm of human chromosome 11 as shown by molecular hybridization studies on somatic cell hybrids containing portions of human chromosome 11 translocated onto other chromosomes.

(3) Physical microinjection can be used to inject biologically active molecules into MEL cells. Techniques were developed to attach tetraploid MEL cells to a coverslip. A tiny volume (10^{-11} ml) of the inducer hexamethylene bisacetamide (HMBA) was microinjected into each attached cell. The injected cells were then shown to differentiate normally into erythroid cells synthesizing hemoglobin. Thus (a) the microinjection technique does not injure the MEL cells and (b) the inducer HMBA appears to act inside the cell rather than on the outside of the cell membrane. This technique has recently been used to introduce intact genes into MEL and other tissue culture cells. The herpes simplex thymidine kinase (TK) gene and the human genomic beta globin gene, both separately and together, have been microinjected into TK$^-$ 2S MEL cells. If this attempt to establish the human globin gene in the MEL cell is successful, it will open the way for studying the integration, replication and transcription of normal as well as abnormal globin genes.

(4) Establishment of a eukaryotic cell-free transcription system using the cloned mouse beta globin gene as template appears to be feasible. Techniques have been developed for obtaining large amounts of the pure mouse beta major globin gene. Known components of the cell's transcription machinery are being purified for use in the cell-free system. This transcription assay will be used to search for and purify globin gene regulatory factors.

(5) Three mouse lines, produced by induced mutations, exhibit a true alpha thalassemia syndrome. Each line has both the adult alpha globin gene and the embryonic alpha-like globin gene inactivated. These thalassemic mice are being studied by DNA restriction endonuclease mapping and by mRNA:cDNA hybridization in order to determine the molecular defect(s) which results in the absence of the alpha globin chain. The long-term objective is to attempt to correct the globin gene defect in these alpha thalassemic mice by insertion of a normal alpha globin gene.

UNIT OF MOLECULAR CLONING

The objective of this Unit is to apply molecular cloning technology to the analysis of genomic DNA sequences involved in the regulation of globin gene expression in humans, sheep and mice.

The Unit has succeeded, during the past year, in:

(1) Establishing and using the in vitro packaging system of bacteriophage lambda for the cloning and isolation of genomic sequences.

(2) Subcloning, into plasmids, various regions of human and mouse genomic DNA from bacteriophage clones. These subclones are being used by both LMH and CHB to facilitate more detailed analysis of globin gene regions which affect transcription and processing of globin mRNA. In addition, genomic plasmid clones, together with other plasmid and bacterial clones containing a number of specific globin gene cDNA sequences, have been prepared for use as hybridization probes in a wide range of experiments carried out in both laboratories.

(3) Creation of a number of recombinant DNA libraries from human thalassemia patients. This project is being carried out in collaboration

with the CHB. From one such library, part of the adult human beta and embryonic beta-like globin gene sequences has been isolated.

(4) Construction of a sheep genomic library and the analysis of sheep globin clones obtained from it. This is a collaborative project with the Clinical Hematology Branch.

(5) Isolation of genomic collagen sequences from the sheep genomic library. This project is being carried out in collaboration with the Pulmonary Branch.

UNIT OF PROTEIN BIOSYNTHESIS

The objectives of this Unit are to: (1) determine the mechanisms involved in translational control of protein synthesis; (2) identify and characterize protein and nucleic acid translational components participating in these regulatory mechanisms; and (3) examine the regulation of globin gene expression at the translational level in normal and disease (e.g., thalassemic) states.

Translational regulation of protein synthesis by hemin, double stranded RNA, and oxidized glutathione appears to involve changes in the phosphorylation state of the protein synthesis initiation factor eIF-2. The Unit has studied factors which control the phosphorylation state of the methionyl-tRNA binding protein eIF-2 and the relationship of eIF-2 phosphorylation to the mechanism of translational inhibition resulting from hemin deficiency.

During the past year the Unit has: (1) developed a sensitive assay for eIF-2α phosphatase activity and studied its regulation by the guanine nucleotide pool in unfractionated lysate; (2) extensively purified and characterized eIF-2α phosphatase; (3) directly measured the eIF-2 pool size and phosphorylation state in lysate; (4) examined the role of low molecular weight metabolites in maintaining catalytic utilization of eIF-2 in lysate; (5) developed large scale preparative procedures for purification of the three eIF-2 subunits; and (6) shown that phosphorylation does not directly inhibit eIF-2 activity, but results in the conversion of eIF-2 from catalytic to stoichiometric utilization. As a result, a greater understanding of the relationship between eIF-2α kinase, eIF-2α phosphatase, the physicochemical state of eIF-2, and the metabolic state of the cell has been achieved. Detailed investigations of each of these four determinants of the eIF-2 phosphorylation state and their relationship to catalytic eIF-2 recycling are being pursued.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 02212-03 MH

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Molecular Control of Eukaryotic Gene Expression

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

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	K. Li	Biologist	MH NHLBI
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	N. Young	Expert	CHB NHLBI
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COOPERATING UNITS (if any)

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LAB/BRANCH
Laboratory of Molecular Hematology

SECTION

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
8.0	2.8	5.2

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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 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 which can turn on the human fibroblast alpha and beta globin genes in MEL x human fibroblast hybrids. The factor(s) is globin gene specific since it will not activate the human gamma globin gene. Attempts are being made to isolate, purify and characterize regulatory factors using the techniques of physical microinjection into intact cells and cell-free transcription systems using cloned globin genes as templates.

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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. Mouse erythroleukemia (MEL) cells are fused by means of inactivated Sendai virus (or polyethylene glycol) to other somatic cells which normally are either capable or incapable of synthesizing hemoglobin. Chromosome and isozyme analysis are done by standard cell biology techniques.

(2) Cell hybrids are analyzed for the ability to synthesize globin messenger RNA (mRNA) or globin. Globin mRNA is detected by hybridization with the appropriate complementary DNA (cDNA); the presence of globin is detected by radioactive leucine incorporation followed by either electrophoresis or column chromatography.

(3) Injection of test material is carried out by physical microinjection into specific regions of intact mammalian cells by using ultrathin micropipets under 200-fold phase optics. Single cells are assayed either by indirect immunofluorescence to detect globin chains or benzidine staining to detect hemoglobin. Single cell clones are grown to 10^8-10^9 cells for isolation of DNA and/or RNA for analysis by restriction endonuclease mapping or by mRNA-cDNA or DNA-cRNA ligand hybridization.

(4) Cloned globin genes are grown, isolated and purified by recombinant DNA techniques.

(5) Components for cell-free transcription are purified by standard DNA, RNA and protein purification techniques.

Major Findings:

(1) The positive regulatory factor(s) identified last year in induced 2S MEL-human fibroblast hybrid cells has now been shown to be globin gene specific. This factor is, therefore, the first gene-specific regulatory factor identified in a eukaryote. In a series of different hybrid cells, the human fibroblast beta globin gene (as well as the human alpha globin and the mouse alpha and beta globin genes) were activated but not the closely linked human gamma globin gene. The factor(s) acts before the pre-mRNA processing step since no gamma globin mRNA sequences were found in the nuclei of the hybrid cells. In addition, several different inducers all resulted in activation of the

factor(s) including, dimethylsulfoxide (DMSO), hexamethylene-bisacetamide (HMBA) and hemin.

(2) The gamma-delta-beta globin gene complex has been mapped to the short arm of human chromosome 11. Cells from patients which contain translocations of either the short arm or the long arm of human chromosome (HC) 11 to portions of either HC15 or HC17 and clones of various human-rodent hybrid lines using these cells were obtained from Dr. Uta Francke, Yale University. Each cell line was expanded to 10^8 cells and the DNA was isolated and annealed to pure human beta globin cDNA probe in order to detect the human beta globin gene. In every cell line containing the short arm of HC 11, the human beta globin gene was present. In every hybrid cell line in which the short arm was absent (even if the long arm of HC 11 was present), the human beta globin gene was absent. Attempts are now being made to lock the short arm of HC 11, attached to a portion of HC17 containing thymidine kinase (TK), into our TK^- 2S MEL cells.

(3) Hybridomas are being developed to obtain monoclonal antibodies to specific human globins as well as to other macromolecules of interest (erythropoietin for the CHB, and later, protein synthesis initiation factors for the Unit of Protein Biosynthesis). Mice are prepared by CHB by immunizing with a specific antigen. Spleen cells are then fused with a myeloma cell line to give a somatic cell hybrid (hybridoma) which secretes monoclonal antibodies to the original antigen. A series of fusions are now underway.

(4) Physical microinjection has been used to introduce biologically active molecules into MEL (and other tissue culture) cells. Last year we showed that fibroblasts could be microinjected with cloned globin cDNA (specifically, the rabbit beta globin cDNA plasmid clone pBG1). The fibroblasts transcribed and translated the globin cDNA genes but at a very low efficiency. We have now developed the technique to attach 2S MEL cells to a coverslip, microinject each cell with approximately 10^{-11} ml of test solution, and grow the injected cells normally. Microinjection of the inducer HMBA produces cells which are induced to make hemoglobin in an apparently identical manner as when the cells are incubated in medium containing the inducer. Thus, (a) the microinjection technique does not injure the MEL cells and (b) the inducer HMBA appears to act inside the cell rather than on the outside of the cell membrane.

(5) The cloned herpes simplex thymidine kinase (TK) gene and the human genomic beta globin gene (HβG1) were prepared (see report # ZO1 HL 02214-02 MH). These full-length intact genes were microinjected either separately or together into attached 2S MEL cells. The objective is to attempt to establish the human globin gene in the MEL cell. If successful, this will open the way for studying the integration, replication and transcription of normal as well as abnormal globin genes in an erythroid environment.

(6) A eukaryotic cell-free transcribing system using a cloned mouse genomic globin gene as template is being established. Milligram quantities of a 7 kilobase DNA fragment encompassing the mouse beta-major globin gene have been obtained by restriction endonuclease digestion of lambda bacteriophage DNA

containing the insert. Purification is by isolating the band containing the globin gene from polyacrylamide gel electrophoresis. Known components of the cell's transcription machinery are being purified for use in this cell-free system. DNA dependent RNA polymerase II, histones and non-histone proteins are being purified from mouse liver. Globin pre-mRNA will be measured by molecular hybridization using pure mouse beta-major globin DNA. Fractions from the nuclei of induced MEL cells will be analyzed in an attempt to isolate the positive regulatory factor(s) that influences globin gene expression in vivo.

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, cancer, etc.

Proposed Course of Project: Fractionation of cell components from induced 2S MEL (and other) cells to identify positive regulatory factors involved in gene expression. Once identified, the factor(s) will be purified and characterized. The biological function of the individual factors will be studied both in regards to their ability to interact with DNA and their ability to control the regulation of gene expression. Microinjection of genomic globin DNA fragments into erythroid and non-erythroid cells will be carried out to study the regulation of globin mRNA transcription.

Publications:

1) Anderson, W. F., Willing, M. C., Axelrod, D. E., Gopalakrishnan, T. V., and Diacumakos, E. G.: A new approach in the search for globin gene regulatory factors. In Stamatoyannopoulos, G., and Nienhuis, A. W. (Eds.): Proceedings of the Conference - Cellular and Molecular Regulation of Hemoglobin Switching. New York, Grune & Stratton, 1978, pp. 779-792.

2) Willing, M. C., Nienhuis, A. W., and Anderson, W. F.: Selective activation of human β- but not γ-globin gene in human fibroblast x mouse erythroleukemia cell hybrids. Nature 277: 534-538, 1979.

3) Gopalakrishnan, T. V., and Anderson, W. F.: Epigenetic activation of phenylalanine hydroxylase in mouse erythroleukemia cells by the cytoplast of rat hepatoma cells. Proc.Natl. Acad. Sci. USA, in press.

4) Diacumakos, E., Killos, L., Lee, L., and Anderson, W. F.: Induction of mouse erythroleukemia cells by microinjection of inducing compound. Science, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 02213-02 MH

PERIOD COVERED
October 1, 1978 to September 30, 1979

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 Fellow	MH NHLBI
	W. Kemper	Chemist	MH NHLBI
	D. Crouch	Biologist	MH NHLBI

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LAB/BRANCH
Laboratory of Molecular Hematology

SECTION

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NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.4	3.0	0.4

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☐ (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. Translational regulation involves changes in the phosphorylation state of the initiation factor eIF-2. We have therefore studied the role of eIF-2 phosphorylation 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. We have identified and characterized four distinct components which regulate the steady state phosphorylation level and activity of the initiator methionyl-tRNA$_f$ binding protein eIF-2, (1) eIF-2α, (2) eIF-2 phosphatase, (3) conformational state eIF-2 and (4) binding of eIF-2 to other translational components. Determination of the eIF-2 pool size, direct chemical measurement of the eIF-2 phosphorylation state, and correlation of these with extent and kinetics of onset of translational inhibition now show that while phosphorylation of eIF-2 does not directly inhibit its activity, phosphorylation is required for additional modification of eIF-2 which results in conversion of catalytic to stoichiometric utilization of eIF-2.

Objectives: The major goals of this project are to (1) determine the sites and mechanisms of translational control of protein synthesis, (2) identify, isolate, and characterize the translational and regulatory components involved in such regulation, and (3) examine the participation of these mechanisms in the regulation of globin gene expression in normal and diseased states. The following areas are being actively investigated: (1) development of rapid and sensitive assay procedures to study the regulation of eIF-2α kinase and phosphatase activities in unfractionated systems; (2) purification and characterization of eIF-2α kinase and phosphatase; (3) examination of eIF-2 sulfhydryl group modification, its effect on methionyl-$tRNA_f$ binding activity, and the relationship to the glucose-6-P dehydrogenase/glutathione reductase system; (4) development of a partially fractionated reticulocyte lysate system which retains high activity and physiologic controls, but which permits experimental alteration of metabolites which affect translational controls; (5) separation and purification of the three eIF-2 subunits, direct determination of their phosphorylation levels and sites in different mechanisms of translational control, and characterization of their partial activities.

Methods: The phosphorylation state of eIF-2α in unfractionated reticulocyte lysate is determined under different metabolic conditions by two-dimensional polyacrylamide gel electrophoresis. The specific activity of ^{32}P in the adenine and guanine nucleotide pools is maintained constant by substrate level phosphorylation according to a modification of the procedure of Glynn and Chappell. Thin layer PEI chromatography is used to measure the specific activities of the nucleotide and phosphate pools. The activity of eIF-2α kinase is monitored as incorporation of ^{32}P into the α subunit of purified eIF-2. [^{32}P]eIF-2α phosphatase activity is measured as loss of TCA precipitable [^{32}P]. Specific preinitiation complexes are obtained from normal and specifically inhibited reticulocyte lysates using sucrose density gradient fractionation. RNA components are quantitatively identified by nucleotide hybridization techniques and cetyltrimethylammonium bromide precipitation of radiolabeled aminoacyl and peptidyl tRNAs. Protein components are identified by comparing direct or autoradiographic visualization of sucrose gradient fractions following resolution by polyacrylamide gel electrophoresis with known translational factors. Purification of translational components present in specific preinitiation complexes is accomplished by ion-exchange column chromatography, glycerol and sucrose density gradient centrifugation, gel filtration, and affinity column chromatography. Biological activity of normal and radiolabeled purified translational components is tested. Standard, mRNA dependent, and other modified reticulocyte lysate preparations, as well as purified components, are being used.

Major Findings:

1) The phosphate content of purified eIF-2 has been determined by direct chemical analysis and is 2 pmol Pi/pmol eIF-2. The increased steady state phosphorylation level of eIF-2 reported in hemin deficient reticulocyte lysates (as determined by incorporation of ^{32}P), therefore, may not reflect an absolute increase in the phosphate content of eIF-2α, but rather an increased rate of phosphate turnover.

2) Restoration of protein synthesis in hemin-deficient lysates by exogenous eIF-2 is stoichiometric. Although the onset of translational inhibition in hemin deficient lysate can be interpreted as a conversion from catalytic to stoichiometric utilization of eIF-2, direct determination of the eIF-2 pool size by isotope dilution (30 pmol/ml of lysate) shows that the amount of eIF-2 in lysate is too small to account for the normal period of protein synthesis in such hemin deficient lysate before the onset of translational inhibition.

3) [^{32}P]eIF-2α phosphatase activity appears to be regulated primarily by alteration of the metabolic state of its substrate (eIF-2), rather than by any direct effect. The energy charge of the guanine nucleotide pool and the redox state of the NADP pool appear to regulate accessibility of the phosphorylated site on eIF-2α to its phosphatase.

4) Sodium selenite inhibits the initiation of protein synthesis by inactivating eIF-2. Similar to the mechanism of translational inhibition resulting from hemin deficiency, the phosphorylation state of eIF-2α appears to be increased; however, neither eIF-2α kinase nor phosphatase activity appear to be directly affected. Covalent modification of a sensitive sulfhydryl group by Na_2SeO_3 does not directly inactivate eIF-2, but does inhibit dephosphorylation by its phosphatase. The possibility that eIF-2 phosphorylation is the first step of a multi-step sequence capable of modifying eIF-2 activity is being investigated.

5) The three subunits of eIF-2 are being individually purified in both native and denatured forms. This should permit clarification of the mechanism of catalytic eIF-2 recycling in reticulocyte lysate.

6) The site of inhibition in the initiation sequence during hemin deficiency translational inhibition appears to be at the point of 60S ribosomal subunit joining. Activation of Met-tRNA$_f$ deacylase may be involved.

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. 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 mechanisms involved in these processes to be able to control gene expression in the cell.

Proposed Course of Project:

The specific kinase and phosphatase which regulate the phosphorylation state of the α subunit of eIF-2 are being purified. This will allow characterization of factors which regulate their activities; in addition, it will now be possible to correlate eIF-2 activity with a specific phosphorylation state. The development of procedures to isolate large amounts of each of the three eIF-2 subunits will also permit amino acid sequencing and identification of the phosphorylation sites, as well as study of their partial activities. The overall goal of these approaches will be to understand the mechanism which normally allows catalytic recycling of eIF-2, and the defect(s) which occurs during hemin deficiency which produced stoichiometric utilization of eIF-2 and translational inhibition. The interaction of this mechanism with the metabolic state of the cell to coordinate translational activity will also be investigated. Although we are presently concentrating on the mechanism of hemin-regulated translational control, preliminary studies of translational activity modifiers from human red blood cells indicate that several other distinct mechanisms for translational control exist. In collaboration with other laboratories, these studies on translational control will be extended into the areas of viral-host interaction, interferon and cell differentiation. As work on insertion of genetic information into cells progresses, we anticipate becoming directly involved in examining the overall control of its expression.

Publications:

1. Safer, B., Jagus, R., and Kemper, W. M.: Analysis of initiation factor function in highly fractionated and unfractionated reticulocyte lysate systems. Methods Enzymol. 60: 61-87, 1979.

2. Goldstein, J., and Safer, B.: Use of heparin-sepharose for the rapid isolation of initiation and elongation factors. Methods Enzymol. 60: 165-181, 1979.

3. Safer, B., and Anderson, W. F.: The molecular mechanism of hemoglobin synthesis and its regulation in the reticulocyte. Crit. Rev. Biochem. 5: 261-290, 1979.

4. Safer, B., and Jagus, R.: Control of eIF-2 phosphatase activity in rabbit reticulocyte lysate. Proc. Natl. Acad. Sci. 76: 1094-1098, 1979.

5. Peterson, D., Merrick, W. C., and Safer, B.: Binding and release of radiolabeled eIF-2 and eIF-3 during 80S initiation complex formation. J. Biol. Chem. 254: 2509-2516, 1979.

6. Jagus, R., and Safer, B.: Quantitation and localization of globin messenger RNA in reticulocyte lysate. J. Biol. Chem., in press.

7. Safer, B., Kemper, W., and Jagus, R.: The use of [^{14}C]eIF-2 to measure the endogenous pool size of eIF-2 in rabbit reticulocyte lysate. J. Biol. Chem., in press.

8. Peterson, D. T., Safer, B., and Merrick, W. C.: Role of eIF-5' in the formation of 80S initiation complexes. J. Biol. Chem., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02214-02 MH

PERIOD COVERED
October 1, 1978 to September 30, 1979

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

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COOPERATING UNITS (if any)
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3.1	2.2	0.9

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)

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, sheep and mice. In collaboration with the Clinical Hematology Branch, the lambda packaging system has been developed and used to create DNA libraries of a number of human thalassemia patients and also a sheep DNA library. Initial screening of one such thalassemic library resulted in the isolation of adult beta and embryonic globin gene sequences. Further, many fragments of DNA relating to globin gene expression in humans, sheep and mice were isolated from genomic clones (constructed in this and other laboratories) and subcloned into plasmids for use by members of both the LMH and CHB.

Studies on the translocation of the ampicillin transposon have been concluded. The inhibitory effect of either temperature of absence of protein synthesis was demonstrated in this system, which may possibly serve as a model for eukaryote gene expression and genome rearrangement.

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Objectives: The objectives of the Molecular Cloning Unit are to establish any apply plasmid, bacteriophage and cosmid recombinant DNA cloning systems for the isolation and analysis of human, mice and sheep genomic DNA sequences involved in the regulation of globin gene expression. Studies concerning the sheep projects are conducted in the Clinical Hematology Branch. In collaboration with the Pulmonary Branch, studies aimed at the isolation and characterization of those genomic DNA sequences responsible for the control of human and sheep collagen synthesis are being carried out. Bacteriophage and plasmids containing cDNA sequences of sheep, human, rabbit and mice isolated in this and other laboratories are being obtained, and DNA isolated from these strains to be used as hybridization probes by members of both the Molecular Hematology Laboratory and the Clinical Hematology Branch. Finally, a bacterial study of parameters affecting translocation of the transposable element, Tn3, is to be concluded in anticipation that the control of gene expression determined by such bacterial insertion sequences will serve as a model system for the control of eukaryotic gene expression.

Methods:

1) Establishment of the in vitro packaging system of bacteriophage lambda. Prior to a cloning experiment, the in vitro packaging system requires that three components be prepared. The first component involves preparation of three bacterial extracts, called protein A, freeze-thaw lysate, and sonic extract, which provide between them all the proteins needed to form bacteriophage particles in vitro. The second component is the preparation of the vector DNA, which in our case is the outer EcoRI "arms" of the vector, charon 4A. The third component is the isolation and purification of 15-20 kb segments of genomic DNA containing EcoRI ends.

The first step in the cloning procedure is to ligate the charon 4A arms with the genomic DNA such that long linear concatemers of DNA result. This in vitrorecombinant DNA can then be efficiently packaged into bacteriophage particles using the bacterial extracts of component 1 (see above), resulting in a cloned library of one million segments of genomic DNA. This library is then screened with radioactively labeled globin sequence by the well-established plaque hybridization method.

2) A number of libraries of thalassemia patients are constructed as described in (1) above, and screened using nick translated beta globin sequence as probe.

3) Approximately 10-20 kilobases (kb) of genomic DNA can be present in any one bacteriophage clone constructed as described above. It is often useful for a number of reasons to subclone much smaller segments of these clones, obtained either in this or other laboratories, into bacterial plasmid vectors. For this reason, the Molecular Cloning Unit maintains stocks of those plasmid vectors such as pBR313, pBR322, pBR325, pACYC184, and pHC79, which may be useful for such subcloning procedures. These plasmid vectors can be 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 of their plasmid DNA on agarose gels.

Major Findings:

1) The in vitro packaging system was established in this laboratory. The efficiency of packaging (1-2 x 10^8 plaque forming units per microgram of lambda DNA) was equal to that reported previously (Blattner et al., Science [1978] 202, 1279-1284). The system has been used reproducibly to create a number of recombinant DNA libraries.

2) A number of genomic DNA fragments have been subcloned into a variety of plasmids. The 7 kb genomic EcoRI fragment containing mouse beta-major globin sequences has been subcloned into the EcoRI site of the plasmid pACYC184. Various sections of this 7 kb fragment have been subcloned into the plasmid pBR322. In addition, various fragments from the human delta and beta genomic region have been subcloned from bacteriophage into pBR322 and pBR325. Similarly, a number of sheep globin sequences have been subcloned into pBR322 and pBR325 (see the Clinical Hematology Branch annual report).

3) In collaboration with the Clinical Hematology Branch, a genomic library of a million unique bacteriophage clones containing DNA from a beta-plus thalassemia patient was constructed and screened for DNA sequences homologous to the adult human beta gene. This screen yielded two clones containing human embryonic DNA, and one clone containing the 3'-end of the adult beta gene. We have recently constructed a new recombinant DNA library of this patient, and are currently screening it for the 5' end of the adult beta gene. In addition, we have constructed a library of a second beta-plus thalassemia patient and will begin screening it for adult beta gene sequences shortly.

4) In a project conducted in the Clinical Hematology Branch, sheep globin sequences have been isolated from a sheep genomic library of one million clones. This work is described in the Clinical Hematology Branch annual report.

5) In collaboration with members of the Pulmonary Branch, part of a sheep library was screened for clones containing sequences homologous to collagen cDNA. Of 150,000 recombinants screened, four were found to contain collagen gene sequences. Characterization of these clones is described in detail in the Pulmonary Branch annual report.

6) Studies which have characterized the effect of temperature and protein synthesis on Tn3 transposition have been concluded. It was shown that both these parameters could probably be utilized to advantage in future studies on mechanisms of translocation of Tn3.

Publications:

1) Kretschmer, P.J., and Cohen, S. N.: Effect of temperature on the translocation frequency of the Tn3 element. J. Bacteriol., in press.

2) For publications relating to sheep, see the Clinical Hematology Branch annual report.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 02215-01 MH

PERIOD COVERED
October 1, 1978 to September 30, 1979

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

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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)

We conclude that three induced mutations in the mouse are true alpha thalassemias. Each has fully inactivated both of the adult alpha genes and the embryonic alpha-like globin gene. Two of the mutations lie at or near the normal Hba alpha globin locus on Chromosome 11. DNA and RNA of heterozygous alpha thalassemics on chosen genetic backgrounds are being analyzed to determine whether each mutation is a chromosomal deletion or a master control mutation.

PHS-6040
(Rev. 10-76)

Objectives: The objective of this work is to characterize fully the nature of the defect which obliterates the expression of alpha globin genes in three induced mouse alpha thalassemias.

Methods:

(1) DNA prepared from genetically-characterized normal and heterozygous alpha thalassemic mice is digested with restriction endonucleases, then transferred to nitrocellulose membrane filters following 0.8% agarose gel electrophoresis. Alpha (or beta) globin genes or fragments are detected by hybridization with probes made by nick-translation of the globin cDNA clones, Charon 3A-117 alpha (or 3A-4beta).

(2) Reticulocyte mRNA, erythroid cell nuclear or cytoplasmic RNA, or genomic DNA is analyzed by liquid hybridization with alpha or beta-specific nick-translated cloned cDNA probes.

(3) Hemoglobins and globins are analyzed by slab polyacrylamide gel isoelectric focusing, electrophoresis, and ion-exchange chromatography.

(4) A simple osmotic resistance test is used to detect thalassemic mice for breeding.

Major Findings:

(1) No detectable adult alpha globin is made from the Hba^{th-J} (Jackson Laboratory) alpha-thalassemic mutant chromosome. Adults heterozygous for this mutation and the 352HB mutation map to Chromosome 11 near the wa-2 locus (i.e., at or near the normal Hba (alpha locus) position). Embryos heterozygous for each of the three alpha thalassemias do not have increased EI hemoglobin (which contains the x (alpha-like) embryonic chain) relative to their other hemoglobins. Therefore, their x loci must be inactive, as well as their adult alpha globin locus. We conclude that the x and Hba loci must be very closely linked on the same chromosome.

(2) In one experiment using mice supplied directly from Oak Ridge, alpha/beta chain synthesis ratios in reticulocytes of mice 352HB and 27HB thalassemia heterozygotes were around 0.9. Mice of defined genotype are being bred here to retest this provocative finding, a surprisingly high value in view of the severity of their blood disease.

(3) DNA from the three lines of thalassemic mice has been obtained which is suitable for Southern transfer and hybridization analysis. Successful transfers and hybridizations have been achieved using mixed cDNA probes and phage-marker probes. Nick-translated cloned alpha globin cDNA is now being prepared for analysis of the genomic DNA.

(4) RNA has been isolated from the reticulocytes, spleen nuclei and spleen cytoplasm, and DNA has been obtained from the liver of all three alpha thalassemic mice. Nick translated single stranded cloned alpha and beta globin cDNA are being prepared in order to measure the absolute amounts of alpha and beta globin genes and mRNA.

(5) We have prepared DNA from our genetically-defined heterozygotes of each of the three mutants with Hba^f. Since the 11 kb genomic alpha fragment characteristic of each of the thalassemic chromosomes prior to mutation is not found in Hba^f mice, these samples will indicate whether that 11 kb piece (thought to carry one of the three entire adult-alpha genes) is present in or deleted from the mutated chromosomes. Normal variants with shifts in the 10 kb, 4.7 kb, and 2 kb alpha bands will be sought among mice which make different assortments of alpha chains (that is, with various Hba genotypes). Four new genotypes have already been found among exotic mice maintained by M. Potter, and homozygous stocks appropriate for DNA analysis are now being created.

Significance to Biomedical Research and Institute Program: These thalassemias are the first mammalian mutations induced in vivo to be analyzed at the genomic DNA level. These thalassemic mice are the best models currently available for a human molecular disease and may be invaluable in the development of methods for the direct therapy of human genetic diseases.

Proposed Course of the Project:

If unusual or seemingly normal alpha globin gene DNA is present on the mutated chromosomes, we shall clone these DNAs for further characterization and sequencing. If the alpha globin genes are simply deleted, then the extents of deletion will be measured.

Publications:

1. Whitney III, J. B., Copland, G. T., Skow, L. C., and Russell, E. S.: Resolution of products of the duplicated hemoglobin alpha-chain loci by isoelectric focusing. Proc. Natl. Acad. Sci. 76: 867-871, 1979.

2. Whitney III, J. B., and Russell, E. S.: Linkage of the embryonic alpha-like and adult alpha globin genes. Proc. Natl. Acad. Sci., in press.

3. Popp, R. A., Skow, L. C., and Whitney III, J. B.: Expression of embryonic hemoglobin genes in alpha-thalassemic and in beta-duplication mice. Ann. N. Y. Acad. Sci., in press.

ANNUAL REPORT OF THE

CLINIC OF SURGERY

NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

October 1, 1978 through September 30, 1979

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.

Evaluation of the Right Ventricular Myocardium in Patients with Congenital Heart Disease: Morphologic and clinical relationships. Clinical studies in patients with valvular heart disease and with congenital heart disease suggest that a "myocardial factor" related to dysfunction of the heart muscle itself is responsible for unsatisfactory postoperative clinical courses in certain patients. Additionally, controversies exist whether to perform palliative operations or anatomically corrective operations upon patients with congenital heart anomalies. The proper timing of operations is questioned as well.

No information is available concerning the time courses of morphological changes of myocardial hypertrophy in humans and the relationships of morphological changes to postoperative clinical results.

The myocardium obtained at operation from 75 patients with right ventricular pressure overload (tetralogy of Fallot physiology) was studied by light and electron microscopy. The patients were divided into four groups: I (36 patients) aged 10 months to 10 years, II (22 patients) aged 11 to 20 years, III (8 patients) aged 21 to 29 years, and IV (9 patients) aged 30 to 53 years.

Half of group III and 78% of group IV patients suffered clinically apparent cardiac failure, major arrhythmias, peri-operative death, and/or late death. Alterations of cardiac hypertrophy and degeneration were severe in both groups of patients. Prominent interstitial fibrosis was observed in groups I, II, III, and IV at frequencies of 19%, 23%, 25%, and 100%, respectively. Myofibrillar lysis, myelin figures, smooth endoplasmic reticulum proliferation, and cell membrane associated spherical microparticles were less common in groups I, II, and III (0% to 25%) than in group IV (67% to 100%). Cellular atrophy, disorganization of cells and myofibrils, lipid accumulation, intracytoplasmic junctions, and thickened basal laminae were frequent in cardiac muscle from groups III and IV.

Thus the degenerative morphological alterations of chronic right ventricular hypertrophy in man appear related to clinical myocardial dysfunction. These relationships have implications for patients' long-term prognoses, the type and timing of their operations, and their requirements for intraoperative myocardial protection.

Based upon our studies, we generalize that patients with congenital heart disease and right ventricular hypertrophy, who are over 15 to 20 years old, may experience a less than optimal postoperative clinical course.

Long-Term Followup and Evaluation of the Hancock "SGP" Prosthesis. The controversy of mechanical vs. bioprostheses for cardiac valve replacement continues. Mechanical heart valves are durable, but require long-term anticoagulants and have a high incidence of thromboembolism; bioprostheses tend to degenerate with time but have a low incidence of emboli without anticoagulation. Since July 1970, the Hancock porcine bioprosthesis has been implanted in 392 patients (491 valves). Of these patients, 271 have had isolated or multiple bioprostheses and 121 have had, in addition to the bioprosthesis, a mechanical valve implanted. Patients return to the NHLBI six months postoperatively to undergo examination and cardiac catheterization. Thereafter, clinic visits are scheduled each year and periodic hospitalizations for late followup are recommended. Major complications are recorded and long-term followup data obtained.

Analysis is here confined to patients receiving one or more bioprostheses, and no mechanical implants, except when considering durability. Patients who had bioprostheses were: MVR = 126; AVR = 72; TVR = 5; AVR-MVR = 21; MVR-TVR = 40; and AVR, MVR, TVR = 7 (346 valves with total followup of 512.8 patient years). Overall early mortality (<30 D) was 6.3%; late was 10.2% with myocardial infarction, congestive failure, and infection being most common causes.

Anticoagulants were not routinely used postoperatively, and in 277 patients early embolic events occurred in 8 (2.9%) and late in 18 (6.9%). Anticoagulation complications were thus largely avoided; such complications occur in about 10% of patients taking Coumadin per year.

Cardiac catheterization has revealed good early hydraulic function of the Hancock bioprosthesis in the atrioventricular position. Late hemodynamic data indicate progressive stenosis or regurgitation in 4 of 10 asymptomatic patients studied 4.5 years after implant. These four patients showed increased mean mitral valve gradients (>5 mm. Hg) or decreased calculated mitral valve area (<0.5 cm^2) compared to their six months study. Three of four symptomatic patients showed similar hemodynamic findings. Progressive prosthetic stenosis, secondary to collagen degeneration and calcification, appears to be the etiology of this finding. Seventy-five patients have had aortic valve replacement with the standard Hancock bioprosthesis at NIH. Average peak systolic gradient was 8.7 mm. Hg for sizes 19-31 valves in 68 patients. Satisfactory hydraulic function was found in the smaller 21-23 mm. standard bioprosthesis, contrary to current opinion.

Valve failures have occurred in six patients (8 valves) in our series. Sixty-nine patients have been followed more than 4.5 years, which represents an 8.7% late failure rate. Failures have occurred 56 to 100 months post implant. Five of these patients were operated upon, three survived. The explanted valves were studied by light, electron and scanning microscopy for anatomical findings as well as physical property changes. Calcified,

immobile leaflets with perivalvular thrombosis rendered one valve stenotic. Leaflet disruption in the other 4 valves allowed them to be regurgitant. One patient had coexistent thrombosis of a heterograft in the tricuspid position. Light and electron miscroscopy disclosed degeneration of collagen fibrils, calcification, lipid and fibrinoid deposition in the valve leaflets. The leaflet surfaces contained red cells, aggregates of fibrin and platelets, giant cells and macrophages, but virtually no host endothelium.

It would appear that valve failure can be expected in most patients after 5-8 years. For this reason, future use of the porcine heterograft will be limited to patients aged 60 years or older. The choice of a mechanical prosthesis for use in younger patients has not yet been made.

The Effects of Dobutamine and Dopamine on Patients with Pulmonary Hypertension. Patients with pulmonary hypertension are a heterogenous group with respect to the etiology of the pulmonary vascular changes. The pulmonary vascular resistance is variable in its capacity to respond to stimuli. In general these patients have difficult postoperative courses, both from a respiratory and a cardiac standpoint. Dobutamine and dopamine are the two main beta stimulators utilized to treat low output postoperatively. Their effects on the pulmonary vascular system have not been evaluated.

A group of postoperative cardiac surgical patients with preoperative pulmonary hypertension were studied. Dose-response curves for both dopamine and dobutamine were developed. Hemodynamic parameters were recorded, and from these the systemic and pulmonary vascular resistances were calculated.

Ten patients with preoperative pulmonary hypertension were evaluated. Pulmonary hypertension is defined as a mean pulmonary artery pressure of 40 mm. Hg or greater. The primary cardiac lesions were congenital and acquired; the majority of patients had rheumatic mitral valvular disease. All operations were performed using a midline sternotomy, and cardiopulmonary bypass was utilized with moderate hypothermia. Myocardial preservation was achieved with topical hypothermia and cold crystalloid cardioplegia. Before discontinuation of cardiopulmonary bypass a left atrial pressure line and a triple-lumen Swan-Ganz catheter were placed. The study began only when the patients course had stabilized, usually on the morning following operation. Dose response curves with both dobutamine and dopamine were developed. Initially dopamine was infused through a central venous line at a rate of 2 mcg/Kg/min. At 15 minute intervals the dosage was increased by 2 mcg/Kg/min until the mean aortic pressure rose 15 mm or the heart rate increased by 30. At each interval hemodynamic variables were recorded which permitted calculation of cardiac index, pulmonary vascular resistance, and systemic vascular resistance.

The study revealed that the two drugs were equally effective in raising the cardiac index and decreasing the systemic vascular resistance. However, only dopamine significantly lowered pulmonary vascular resistance. Pulmonary artery pressures increased with both drugs; this resulted solely from increased pulmonary blood flow. Thus, we conclude that the use of dopamine is the drug of choice in treating these patients postoperatively.

Myocardial Preservation. Long-term Effects of Different Forms of Myocardial Protection. Myocardial preservation during cardiopulmonary bypass (CPB) operations with ischemic aortic cross clamp continues to be a topic of clinical and experimental investigations. We and others have investigated the acute hemodynamic effects of several methods currently being used to protect the myocardium including coronary perfusion, hypothermic ischemic arrest, and potassium-based cardioplegia. However, to date, results of chronic studies evaluating different methods of myocardial preservation with reference to left ventricular function and pathology are inconclusive. We have investigated the acute and chronic pathologic and hemodynamic effects of several different methods of myocardial preservation.

Foxhounds weighing 20-30 Kg were divided into the following groups:

Group 1 - Normal control (7 dogs) - Sixty minutes of beating nonworking cardiopulmonary bypass (CPB) at 30° C core temperature without aortic crossclamping.

Group 2 - Ischemic arrest (8 dogs) - Sixty minutes of CPB at 30° with aortic crossclamping.

Group 3 - Cardioplegic Arrest 30° C (6 dogs) - Sixty minutes of CPB at 30° C with aortic crossclamping and potassium-based cardioplegia at 30° C every 15 minutes by aortic root injection.

Group 4 - Cardioplegic Arrest 4° C - Sixty minutes of CPB at 30° C with aortic crossclamping and potassium-based cardioplegia at 4° C every 15 minutes by aortic root injection.

Group 5 - Normal saline 4° C - Sixty minutes of CPB at 30° C with aortic crossclamping and normal saline "cardioplegia" at 4° C every 15 minutes by aortic root injection.

All animals undergo cardiac catheterization pre- and postoperatively to determine baseline and serial left ventricular function. Cardiac enzyme determinations are made preoperatively, immediately upon separation from CPB, and on postoperative day number one. All dogs are being followed six months with postoperative catheterizations.

Significant preliminary data from Groups 1, 2, and 3 reveal the following:

1) Baseline left ventricular function is similar in the three groups as measured by left ventricular function curves, dp/dt max, Vpm (Vce max during isovolumic systole), pulmonary wedge pressure, and cardiac output.

2) Group 1 dogs all weaned from CPB without clinical evidence of ventricular depression. There was no need for pressor support.

3) Group 2 dogs required pressor support.

4) Group 2 dogs had significantly elevated CPK-isoenzymes at postoperative day one ($p < .003$).

5) Groups 1 and 2 were both functionally depressed at postoperative day one, but returned to preop values by one week postop.

6) Group 3 dogs had no significant functional depression of Vpm, dp/dt, cardiac output, or left ventricular function curve either acutely or at the six month catheterization. These dogs weaned from CPB without pressor support.

7) Group 3 dogs had no CPK-isoenzyme elevation compared with either Group 1 or 2 ($p < .002$).

Data collection on the remaining groups of animals will be continued during the upcoming academic year.

Late Results Following Operative Correction of Congenital Aortic Valvular Stenosis. Congenital aortic stenosis comprises 3-5% of all congenital heart disease. Left ventricular obstruction at the valvular level is the most common form. We have reviewed 47 patients who underwent aortic valvotomy for congenital valvular aortic stenosis.

Between 1958 and 1974, 47 patients, ages 1-31 years, with congenital aortic valvular stenosis underwent aortic valvotomy; there were no operative deaths. At the time of operation 45 patients had bicuspid valves, and 2 patients had unicuspid valves; 3 patients had hypoplastic outflow tracts. Forty-five patients have been followed postoperatively for 5-21 years (mean 13 years). Currently 67% (30/45) are asymptomatic. Aortic regurgitation is evident in 22 patients, but in 14 of them the diastolic murmur is only grade I-II/VI. Postoperative cardiac catheterization has been performed in 38 patients. The average peak systolic valve gradient was 30 mm. Hg; the average aortic valve index (17 patients) was 0.66 cm^2/M^2. Seven of 45 patients (16%) have required aortic valve replacement from 5-17 years (mean 11 years) postoperatively. Three of 45 patients (7%) required treatment of bacterial endocarditis. Aortic valvotomy provides satisfactory palliation for patients with congenital valvular aortic stenosis, but late valve-related complications mandate continuing patient observations. Valve replacement will probably be necessary in all patients at some interval after valvulotomy.

Regional Myocardial Blood Flow in Left Ventricular Hypertrophy. Vulnerability of the subendocardium to ischemic injury has been documented during a variety of physiologic conditions in animals with normal hearts and those with left ventricular hypertrophy secondary to chronically elevated afterload distal to the coronary arteries. However, the lack of a laboratory model for left ventricular hypertrophy with aortic valve or subvalve stenosis has restricted investigation of regional myocardial blood flow in this setting. To study this, we subjected Newfoundland dogs with congenital discrete suboartic stenosis and left ventricular hypertrophy to a variety of hemodynamic alterations while measuring regional myocardial blood flow with radioactive microspheres.

Eleven dogs with LVH, left ventricle to body weight ratio (LV/BW)= $6.35 \pm .46$ Gm/Kg (mean $\pm$ SEM), were compared to 12 dogs without LVH,

LV/BW = 3.41 ± .12 Gm/Kg. The dogs were subjected to atrial pacing (221 ± 4/min), ascending aortic constriction producing systolic hypertension (206 ± 5 mm.Hg), and creation of an aorto-right atrial fistula producing diastolic hypotension (38 ± 3 mm.Hg). LV endocardial to epicardial flow ratios were:

	NORMAL	LVH
CONTROL	.97±.04	.91±.05
PACED	.96±.04	.59±.07*
CONSTRICTION	.95±.04	.70±.06*
FISTULA	.86±.08	.57±.08#

* $p < .005$ and # $p < .02$ for LVH compared to normal

This study is the first one using a model for LVH with infracoronary obstruction to demonstrate an increased subendocardial vulnerability to ischemic injury in the presence of LVH.

Computerized Acquired and Congenital Cardiac Data Base. A computerized data base had been developed since 1975, which contains all hemodynamic and angiographic data, operations performed, survival statistics and major complications following either corrective or palliative operations for various types of acquired and congenital heart diseases. The data base contains approximately 3700 patients operated upon since the Clinic of Surgery NHLBI was founded in 1953. The Cardiology Branch is currently entering preoperative patients in an expanded data base which is being integrated with Surgery data base.

All patient's charts have been reviewed to retrieve cardiac catheterization data, operative diagnoses and procedure performed, postoperative followup, including major complications and causes of death and long-term functional class profile. Currently, the Cardiology Branch is entering the preoperative patients seen on their service as well as those patients that have died to construct a "natural history" profile prior to operative intervention. Their data base includes EKG, X-ray, ECHO data, as well as the patient's functional class, symptoms, and medications. This data base has been integrated with the Surgery Branch data base for patients with IHSS and all patients who have been operated upon over the past year.

The data base has accomplished a number of important points relative to following pre- and postoperative patients. Clinic charts containing much data not relative to long-term followup, have been condensed to several computer printout sheets containing pertinent data on each patient. These sheets are used for reviews for studies as well as seeing patients in the Outpatient Department or those admitted to 6-West.

In addition to record keeping, the system can be queried for specific subgroups of patients to answer clinical questions regarding specific valve types or operations performed. The system allows limits to be set and variables to be defined allowing selection of cohorts of patients to be studied. Survival curves and lists including deaths and causes, complications hemodynamic summaries and current status may be generated for the various groups of patients.

With the addition of the expanded data profile generated by Cardiology, a complete pre- and postoperative natural history profile is being developed for the various forms of acquired and congital heart disease.

The cardiac data base has been queried approximately 50 times the past year for the purpose of either data collection for publications or for locating specific patients for ongoing studies at the NHLBI. The system is also used weekly to obtain printouts for Clinic and ward patients.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02665-02 SU

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Myocardial Preservation. Long-term Effects of Different Forms of Myocardial Protection.

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

PI: John H. Bell, M.D., Research Associate, Clinic of Surgery

OTHER: William C. Scott, M.D., Clinical Associate in Surgery, Clinic of Surgery
Richard J. Shemin, M.D., Clinical Associate in Surgery " "
Michael Jones, M.D., Senior Surgeon, Clinic of Surgery
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, and Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6	5	1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Myocardial preservation during cardiopulmonary bypass (CPB) operations with ischemic aortic crossclamp continues to be a topic of clinical and experimental investigations. Our group and others have investigated the acute hemodynamic effects of several methods currently being used to protect the myocardium including coronary perfusion, hypothermic ischemic arrest, and potassium-based cardioplegia. However, to date, results of chronic studies evaluating different methods of myocardial preservation with reference to left ventricular function and pathology are inconclusive and not amenable for comparison.

Our laboratory has ongoing experimental projects investigating the acute and chronic pathologic and hemodynamic effects of several different methods of myocardial preservation.

PHS-6040
(Rev. 10-76)

DESCRIPTION: Foxhounds weighing 20-30 Kg are divided into the following groups:

Group 1 - Normal control (7 dogs): Sixty minutes of beating nonworking CPB at 30° C systemic without aortic crossclamping.

Group 2 - Ischemic arrest (8 dogs): Sixty minutes of CPB at 30° C systemic with aortic crossclamping.

Group 3 - Cardioplegic arrest 30° C (6 dogs): Sixty minutes of CPB at 30° C systemic with aortic crossclamping and potassium-based cardioplegia at 30° C every 15 minutes per aortic root injection.

Group 4 - Cardioplegic Arrest 4° C: Sixty minutes of CPB at 30° systemic with aortic crossclamping and potassium-based cardioplegia at 4° every 15 minutes per aortic root injection.

Group 5 - Normal saline 4° C: Sixty minutes of CPB at 30° C systemic with aortic crossclamping and normal saline 'cardioplegia' at 4° C every 15 minutes per aortic root injection.

All animals undergo cardiac catheterization pre- and postoperatively to determine baseline and serial left ventricular function parameters. Cardiac enzyme determinations are made preoperatively, immediately upon separation from CPB, and on postoperative day number one. All dogs are being followed six months with postoperative catheterizations. At six months postoperative, all dogs will be euthanized and the left ventricles will be submitted for gross and histoligic pathological evaluation.

RESULTS; Significant prelimiary date from Groups 1, 2, and 3 reveal the following:

(1) Baseline left ventricular function is similar in the three groups as measured by left ventricular function curves, dp/dt max, Vpm (Vce max during isovolumic systole), pulmonary wedge pressure, and cardiac output.

(2) Group 1 dogs all weaned from CPB without clinical evidence of ventricular depression. There was no need for pressor support.

(3) Group 2 dogs required pressor support.

(4) Group 2 dogs had significantly elevated CPK-isoenzymes at postoperative day one ($p < .003$).

(5) Groups 1 and 2 were both functionally depressed at postoperative day one but returned to preop values by one week postop.

(6) Group 3 dogs had no significant functional depression of Vpm, dP/dT, cardiac output, or left ventricular function curve either acutely or at the six month catheterization. These dogs weaned from CPB without pressor support.

(7) Group 3 dogs had no CPK-isoenzyme elevation compared with either Group 1 or 2 ($p < .002$).

PROPOSED COURSE: Data collection on the remaining groups of animals will be continued during the upcoming academic year. Upon completion of the above listed groups, animals with experimentally created left ventricular hypertrophy will undergo similar evaluation of myocardial preservation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02667-01 SU

PERIOD COVERED

October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Results of the Operation for Idiopathic Hypertrophic Subaortic Stenosis (IHSS): What the Primary Physician Should Know.

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

PI: Barry J. Maron, M. D., Senior Investigator, Cardiology Branch

OTHER: Jean-Paul Koch, M. D., Clinical Associate in Surgery
Kenneth M. Kent, M.D., PhD., Chief Cardiovascular Diagnosis Section
Stephen E. Epstein, M.D., Chief, Cardiology Branch
Andrew G. Morrow, M.D., Chief, Clinic of Surgery

COOPERATING UNITS (if any)

Cardiology Branch, NHLBI

LAB/BRANCH

Clinic of Surgery

SECTION

INSTITUTE AND LOCATION

National Heart, Lung & Blood Institute

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)

From 1960 to April 1979, 233 patients have been operated upon, in the Clinic of Surgery, NHLBI, for relief of symptomatic idiopathic hypertrophic subaortic stenosis. The present study reviews 217 patients who, from April 1960 to May 1978, had at least one year followup.

PHS-6040
(Rev. 10-76)

DESCRIPTION: At operation, a wedge of hypertrophic ventricular septum is resected to relieve left ventricular outflow tract obstruction. Operative mortality has been low (8%). Most patients have had detailed followup, including cardiac catheterization and regular outpatient visits. Over 70% of the patients have had a distinct long-term imporvement, only 11% being not imporved by the operation. Failure to improve in the latter group seems to be due mainly to the underlying cardiomyopathy rather than to failure to relieve their left ventricular outflow tract obstruction. Operative mortality was 8%, late IHSS-related deaths being about 6% and non-IHSS related deaths about 5%.

RESULTS: As a result of this extensive followup we believe that left ventricular myotomy and myectomy is indicated for patients who are severely limited by their disease and whose symptoms do not respond to medical therapy. The operation carries a low mortality rate and good symptomatic improvement.

PROPOSED COURSE: This material has been submitted for publication to Cardiovascular Medicine.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02668-01 SU

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Left ventricular myotomy and myectomy as a proposed treatment for patients with idiopathic hypertrophic subaortic stenosis (IHSS) and preoperative sudden death.

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

PI: Jean-Paul Koch, M.D., Clinical Associate, Clinic of Surgery, NHLBI

OTHER: Barry J. Maron, M.D., Senior Investigator, Cardiology Branch, NHLBI
Kenneth M. Kent, M.D., PhD., Chief, Cardiovascular Diagnosis Section
Stephen E. Epstein, M.D., Chief, Cardiology Branch, NHLBI
Andrew G. Morrow, M.D., Chief, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)
Cardiology 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	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

From 1960 to date 245 patients have been operated upon in the Surgery Branch, NHLBI, for relief of symptomatic IHSS. In a small subgroup of patients, preoperative sudden death has been the major indication for left ventricular myotomy and myectomy.

748

PHS-6040
(Rev. 10-76)

DESCRIPTION: Left ventricular myotomy and myectomy has been recognized as being the treatment of choice for symptomatic idiopathic hypertrophic subaortic stenosis, unresponsive to medical therapy. Whereas in most patients, symptoms include syncope, angina, dyspnea on exertion, orthopnea, fatigue, in a small subgroup of patients, consisting of less than 4% of our total patient population (10 patients out of 245) sudden death with proven ventricular fibrillation was the major catastrophic event which was the main, if not only, indication for operation. This subgroup of high-risk patients will be analyzed as far as personal history, family history, cardiology workup and postoperative course is concerned. All of these patients have had extensive followup, including cardiac catheterization postoperatively and regular outpatient clinic visits. So far there has been one operative death and one late postoperative death, both due to ventricular fibrillation.

RESULTS: The natural history of this group of patients being unknown and sudden death being the main, if not only, preoperative event postoperative results may be very difficult to interpret. Nevertheless, given our experience with the proposed operation and low mortality rates, surgical relief of the left ventricular outflow tract obstruction might be the only alternative for these high risk patients.

PROPOSED COURSE: Continue the study and submit for publication.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02669-01 SU

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Operative treatment of patients 65 years or older with obstruction cardiomyopathy.

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

PI: Jean-Paul Koch, M.D., Clinical Associate, Clinic of Surgery, NHLBI

OTHER: Barry J. Maron, M.D., Senior Investigator, Cardiology Branch, NHLBI
Kenneth M. Kent, M.D., PhD, Chief, Cardiovascular Diagnosis Sec. NHLBI
Stephen E. Epstein, M.D., Chief, Cardiology Branch, NHLBI
Andrew G. Morrow, M.D., Chief, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)
Cardiology Branch NHLBI

LAB/BRANCH
Clinic of Surgery, NHLBI

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, & Blood Institute

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)

Left ventricular myotomy and myectomy for relief of obstructive cardiomyopathy has been a major interest of the Clinic of Surgery since 1960. More than 245 have been operated upon in this Institute between 1960 and May 1979. Post-operative followup has been extensive including cardiac catheterization for postoperative evaluation and frequent outpatient visits. To date 20 patients 65 years of age or older have had left ventricular myotomy and myectomy. The purpose of this study is to review our experience in this particular group of patients traditionally considered as being high-risk.

750

DESCRIPTION: Left ventricular myotomy and myectomy, on cardiopulmonary bypass, is the operation of choice to relieve symptomatic obstructive idiopathic hypertrophic subaortic stneosis, unresponsive to medical therapy. To date 245 patients have been operated upon in this Institute with an operative mortality of 8% and 217 patients had at least one year followup. Seventy percent of all the patients had long-term improvement of at least one functional class (NYHA), only 11% being unimproved by the operation. Between 1972 and 1979, 20 patients 65 years of age or older were operated upon (mean age: 68, the oldest patient being 76). There was one operative death (5%) and one late postoperative death due to myocardial infarction. At six months followup, 19 out of the 20 patients were functionally improved, later followup showing improvement in 12 out of 13 patients of at least one functional class (NYHA). In this patient group, intercurrent disease in more than 50% of the patients will be of major importance as far as long-term functional improvement is concerned.

RESULTS: At a time where more and more older patients are considered for open heart operation, left ventricular myotomy and myectomy is the treatment of choice of symptomatic IHSS, unresponsive to medical therapy. The operation carries a low mortality rate, even in this group of patients, traditionally considered as being poor risk and gives symptomatic relief and improvement in the quality of life, even it it doesn't give immortality.

PROPOSED COURSE: Continue the study and submit for publication.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02670-01 SU

PERIOD COVERED

October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Computerized Acquired and Congenital Cardiac Data Base

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

PI: Charles L. McIntosh, M.D., Ph.D., Senior Surgeon, Clinic of Surgery, NHLBI

OTHER: None

COOPERATING UNITS (if any)

Division of Computer and Research Technology

LAB/BRANCH

Surgery

SECTION

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)

A computerized data base had been developed since 1975, which contains all hemodynamic and angiographic data, operations performed, survival statistics and major complications following either corrective or palliative operations for various types of acquired and congenital heart diseases. The data base contains approximately 3700 patients operated upon since the Clinic of Surgery, NHLBI, was founded in 1953. The Cardiology Branch is currently entering preoperative patients in an expanded data base which is being integrated with the Surgery data base.

752

Description: A computerized data base has been developed in cooperation with DCRT for approximately 3700 patients who have undergone operative intervention for various types of acquired or congenital heart lesions. All patient's charts have been reviewed to retrieve cardiac catheterization data, operative diagnoses and procedure performed, postoperative followup, including major complications and causes of death and long-term functional class profile. Currently, the Cardiology Branch, NHLBI, is entering the preoperative patients seen on their service as well as those patients that have died to construct a "natural history" profile prior to operative intervention. Their data base includes EKG, X-ray, ECHO data, as well as the patient's functional class, symptoms, and medications. This data base has been integrated with the Surgery Branch data base for patients with IHSS and all patients who have been operated upon over the past year.

Results: The data base has accomplished a number of important points relative to following pre- and postoperative patients. Clinic charts containing much data not relative to long-term followup, have been condensed to several computer printout sheets containing pertinent data on each patient. These sheets are used for reviews for studies as well as seeing patients in the Outpatient Department or those admitted to 6-West.

In addition to record keeping, the system can be queried for specific subgroups of patients to answer clinical questions regarding specific valve types or operations performed. The system allows limits to be set and variables to be defined allowing selection of cohorts of patients to be studied.

Survival curves and lists including deaths and causes, complications, hemodynamic summaries and current status may be generated for the various groups of patients.

With the addition of the expanded data profile generated by Cardiology, a complete pre- and postoperative natural history profile is being developed for the various forms of acquired and congenital heart disease.

The cardiac data base has been queried approximately 50 times the past year for the purpose of either data collection for publications or for locating specific patients for ongoing studies at the NHLBI. The system is also used weekly to obtain printouts for clinic and ward patients.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02671-01 SU

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Long-Term Followup and Evaluation of the Hancock "SGP" Prosthesis

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

PI: Charles L. McIntosh, M.D., Ph.D., Senior Surgeon, Clinic of Surgery, NHLBI

OTHER: Kenneth Kent, M.D., Ph.D., Chief, Cardiovascular Diagnosis Section, Cardiology Brancy, NHLBI
Stephen E. Epstein, M.D., Chief, Cardiology Branch, NHLBI
Andrew G. Morrow, M.D., Chief, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)
Cardiology Branch, NHLBI

LAB/BRANCH
Surgery

SECTION

INSTITUTE AND LOCATION
NHLBI-NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6	6	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Since July 1970, 491 Hancock "SGP" bioprostheses have been implanted in 392 patients with 69 patients followed more than four years. Followup data includes early and late mortality and causes, thromboembolic evidence, valve failures, six months postoperative hemodynamic data for atrioventricular and aortic implants, late (7 4.5 years) hemodynamic data for mitral valve patients, and complications following valve replacement with the "SGP" bioprosthesis. Durability of the bioprosthesis implanted more than 4.5 years is marginal. Long-term followup reveals an 8.7 percent valve failure after 4.5 years and late hemodynamic findings consistent with prosthetic stenosis or regurgitation in 4 of 10 patients. All other factors continue to be favorable regarding the Hancock "SGP" bioprosthesis.

754

Description: The controversy of mechanical vs. bioprostheses for cardiac valve replacement continues. Mechanical heart valves are durable, but require long-term anticoagulants and have a high incidence of thromboembolism; bioprostheses tend to degenerate with time but have a low incidence of emboli without anticoagulation. Since July 1970, the Hancock "SGP" bioprosthesis has been implanted in 392 patients (491 valves). Of these patients, 271 have had isolated or multiple bioprostheses and 121 have had, in addition to the bioprosthesis, a mechanical valve implanted. Patients return to the NHLBI six months postoperatively to undergo examination and cardiac catheterization. Thereafter, clinic visits are scheduled each year and periodic hospitalizations for late followup are recommended. Major complications are recorded and long-term followup data obtained.

Results: Analysis will be confined to patients receiving one or more bioprostheses, and no mechanical implants except when considering durability. Patients were implanted with bioprostheses as follows: MVR = 126; AVR = 72; TVR = 5; AVR-MVR = 21; MVR-TVR = 40; and AVR, MVR, TVR = 7 (346 valves with total followup of 512.8 patient years). Overall early mortality ($\leq$ 30 D) was 6.3%; late was 10.2% with myocardial infarction, congestive failure, and infection being most common causes.

Anticoagulants were not routinely used postoperatively, and in 277 patients early embolic events occurred in 8 (2.9%) and late in 18 (6.9%). Anticoagulation complications are thus avoided which may occur in 5% of patients taking Coumadin per year.

Cardiac catheterization has revealed good early hydraulic function of the Hancock "SGP" bioprosthesis in the atrioventricular position. Late hemodynamic data indicate progressive stenosis or regurgitation in 4 of 10 asymptomatic patients studied 4.5 years after implant. These four patients showed increased mean MVG of 5 mm. Hg or decreased calculated MVA of 0.5 cm^2 compared to their six months study. Three of four symptomatic patients showed similar hemodynamic findings. Progressive prosthetic stenosis secondary to collagen degeneration and calcification appears to be the etiology of this finding. Seventy-five patients have had aortic valve replacement with the standard Hancock bioprosthesis at NIH. Average peak systolic gradient was 8.7 mm.Hg for sizes 19-31 valves in 68 patients. Satisfactory hydraulic function was found in the smaller 21-23 mm. standard bioprosthesis contrary to current opinion.

Valve failures have occurred in six patients (8 valves) in our series. Sixty-nine patients have been followed more than 4.5 years which represents an 8.7% late failure rate. Failures have occurred 56 to 100 months post implant. Five of these patients were operated upon, three survived. The explanted valves were studied by light, electron and scanning microscopy for anatomical findings as well as physical property changes. Collagen degeneration, calcification and thrombosis appear to be most common causes for failures.

Other pertinent findings regarding the Hancock bioprosthesis include no significant hemolysis, resistance to systemic infection, and acceptable mode of failure.

Course: This information was presented at the 64th Annual Clinical Congress of the American College of Surgeons, October, 1978, at an International Meeting in Barcelona, Spain, June 1979, and is being submitted for publication.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02672-01 SU

PERIOD COVERED October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Hemodynamic Results Following Aortic Valve Replacement with the Hancock Porcine Heterograft

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
Lewis C. Lipson, M.D., Senior Investigator, Cardiology Branch, NHLBI

OTHER: Harry Michael Lewis, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Douglas R. Rosing, M.D., Senior Investigator, Cardiology Branch, NHLBI
Kenneth M. Kent, M.D., Ph.D., Chief, Cardiovascular Diagnosis Section, NHLBI
Charles L. McIntosh, M.D., Ph.D., Senior Surgeon, Clinic of Surgery, NHLBI
Andrew G. Morrow, M.D., Chief, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)
Cardiology Branch, NHLBI

LAB/BRANCH
Surgery

SECTION

INSTITUTE AND LOCATION
NHLBI-NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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)

Freedom from long-term anticoagulation and a low incidence of thromboembolism have provided the porcine valve heterograft with distinct advantages over mechanical valve prostheses. Recent reports, however, have demonstrated unfavorable hemodynamic function, particularly among the smaller prosthetic sizes. To assess this problem we studied 63 patients following aortic valve replacement with the Hancock porcine heterograft by cardiac catheterization both at rest and conditions of elevated cardiac output by isoproterenol infusion.

PHS-6040
(Rev. 10-76)

Description: Sixty-three patients who underwent aortic valve replacement with the Hancock porcine heterograft returned for routine evaluation six months following operation.. Right and left heart catheterizations were performed with either transseptal or left ventricular puncture in all patients. Fifteen patients received isoproterenol infusion to elevate cardiac output. Simultaneous left ventricular and systemic artery pressures were recorded and cardiac outputs were determined by green dye. Effective valve orifice was computed using the Gorlin formula. Average resting peak gradient, 8 $\pm$ 1 mm. Hg (mean $\pm$ sem), was similar for sizes 21 through 27 mm. Aortic valve areas (AVA) computed by the Gorlin formula were 1.27 $\pm$ .17, 1.46 $\pm$ .11, 11.72 $\pm$ .20 and 1.97 $\pm$ .06 cm^2 for 21, 23, 25 and 27 mm. valves, respectively ($p < .04$). Cardiac and aortic valve indices were not significantly different among various valve sizes. AVA were 76% of the maximal effective orifice area determined by in vitro planimetry. Elevating cardiac output by isoproterenol infusion (15 patients) from 5.5 $\pm$ .3 to 9.0 $\pm$ .5 L/min resulted in an increase in peak gradient from 10 to 44 mm. Hg (range 10-85) independent of prosthesis size ($p < .0001$). Hemodynamic function of the HPH was found to be satisfactory at rest, but significant obstruction may occur when cardiac output is raised.

Proposed Course: This work has been submitted for presentation at the 1979 scientific session of the American Heart Association.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02673-01 SU

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Late Failures of Hancock Porcine Heterografts Used for Mitral Valve Replacement

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
Jeffrey M. Isner, M.D., Staff Associate, Pathology Branch, NHLBI

OTHER: Charles L. McIntosh, M.D., Ph.D., Senior Surgeon, Clinic of Surgery, NHLBI
Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI
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COOPERATING UNITS (if any)

Pathology Branch, NHLBI

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)

The gluteraldehyde stabilized porcine heterograft has become an increasingly popular cardiac valve substitute in spite of its unknown durability. Degenerative changes have been shown by light and electron microscopy to occur in heterograft valve leaflets after variable durations of implantation. Reports of clinical primary valve failure in the absence of infection have been few. We have reviewed the clinical and pathological data from five cases of mitral porcine valve heterograft degeneration.

PHS-6040
(Rev. 10-76)

Description: Fifty-three of 87 patients who underwent mitral valve replacement (MVR) before July 1974 with the Hancock porcine heterograft have been followed for 4.5 to 8.8 years after operation. Five of these patients (9.4%) have suffered valve failure between 56 and 94 months after operation. All 5 patients were NYHA functional Class II prior to developing congestive heart failure (CHF) and new cardiac murmurs. Echocardiograms predicted valve dysfunction in two patients. One patient died from severe CHF and terminal sepsis; the other four underwent reoperation; two died. Radiographic calcification was present in all five failed valves. Calcified, immobile leaflets with perivalvular thrombosis rendered one valve stenotic. Leaflet disruption in the other four valves allowed them to be regurgitant. One patient had coexistent thrombosis of a heterograft in the tricuspid position. Light and electron microscopy disclosed degeneration of collagen fibrils, calcification, lipid and fibrinoid deposition in the valve leaflets. The leaflet surfaces contained red cells, aggregates of fibrin and platelets, giant cells and macrophages, but virtually no host endothelium. In view of these findings, close long-term followup is warranted for patients following MVR with porcine heterografts.

A change in functional status with the appearance of a new cardiac murmur was indicative of porcine valve heterograft failure. The onset of symptoms tends to be insidiously progressive rather than catastrophically sudden as in the case with a mechanical prosthesis. Valve failure occurring in the absence of infection may be regurgitant from leaflet disruption or stenosis from calcification and thrombosis. Both collagen breakdown and calcification appear to be the primary mode of failure. The absence of most endothelium on the graft leaflets permits accumulation of red cells and aggregates of fibrin and platelets.

Proposed Course: This work has been submitted as an abstract to be considered for presentation at the 1979 scientific session of the American Heart Association.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02674-01 SU

PERIOD COVERED

October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Changes in Cardiac, Cutaneous and Splanchnic Sympathetic Activity in Response to Surgical and Anesthetic Stress During and Post Open Heart Surgery for Coronary Artery Bypass Grafts and Valve Replacement.

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

PI: Young D. Kim, M.D., Anesthesiologist, Anesthesiology Department, CC

OTHER: Jean-Paul Koch, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Michael Jones, M. D., Senior Surgeon, Clinic of Surgery, NHLBI
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Virginia Weise, Investigator, Lab of Clinical Science, NIMH
I. J. Kopin, M.D., Laboratory of Clinical Science, NIMH

COOPERATING UNITS (if any)

Department of Anesthesiology, CC and Lab of Clinical Science, NIMH

LAB/BRANCH

Clinic of Surgery

SECTION

INSTITUTE AND LOCATION

National Heart, Lung and Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	3/4	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)

The sympathetic nervous system will be studied in 20 patients undergoing coronary artery bypass grafts (CABG) or valve replacement by simultaneous measurements of plasma levels of epinephrine and norepinepherine from various vascular beds. The changes in the regional SNS activity will be related to simultaneous hemodynamic and metabolic changes.

761

PHS-6040
(Rev. 10-76)

DESCRIPTION: Changes in plasma catecholamine levels reflect SNS activity. However, difficulty has been encountered to explain the changes in hemodynamic and metabolic parameters in relation to the changes in catecholamines obtained from one vascular bed. A sensitive and specific radioenzymatic assay of catecholamines has recently been developed. Utilizing this method, it has been shown that SNS activity of individual organsystems could be assessed by simultaneous measurements of catecholamine levels in systemic arterial and venous blood of a specific organ. In this proposed study, simultaneous measurement of catecholamine levels of the blood from cutaneous vein, peripheral artery, pulmonary artery and coronary sinus will provide an index of regional SNS activity: cutaneous tissue (arterial-cutaneous venous difference), myocardium (arterial-CS difference) and possibly splanchnic organs (mixed venous cutaneous and CS difference). Blood samples will be obtained before induction, after sternotomy, after coming off CPB and in the intensive care room from an arterial line, peripheral vein, Swan-Ganz catheter and coronary sinus line and will be analyzed for catecholamines and correlated to the hemodynamic and metabolic status.

PROPOSED COURSE: The study is in progress.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02675-01 SU

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
An animal model for creating and maintaining long-term patency of the ductus arteriosus

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

PI: John S. Pfeifer, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Richard E. Michalik, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Clinic of Surgery

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	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)

This study was undertaken to evaluate an animal model for maintaining long-term patency of the ductus arteriosus. The adventitia of the ductus arteriosus of newborn lambs was infiltrated with either gluteraldehyde or formalin to maintain patency after prostaglandin E_1 was infused to reopen the ductus which was assumed to be functionally closed in the first few hours after birth. The lambs were then studied at various time intervals both with hemodynamic measurements and radiographically for determination of ductus patency.

PHS-6040
(Rev. 10-76)

DESCRIPTION: There are certain infants with congenital cardiac defects which depend upon patency of the ductus arteriosus for survival. These include those infants with pulmonary atresia, tricuspid atresia with infant ventricular septum, transposition of the great vessels with inadequate mixing or interrupted aortic arches. Despite hypoxemia accompanying these defects, the ductus usually begins to constrict within hours after birth leading to more marked hypoxemia and attendant acidosis, resulting in deterioration of the infant. Some investigators have used prostaglandin E_1 to maintain ductal patency while others have injected the adventitia of the ductus with formalin.

Newborn lambs underwent left thoracotomy 6-18 hours after birth. A cannula was placed in the transverse aortic arch through which prostglandin E_1 was infused as well as central aortic pressure was recorded. The ductus was then exposed by developing a pleural flap through a longitudinal incision in the pleura over the descending aorta. The animals then underwent infiltration of their ductus adventitia with either formalin or 1.5% gluteraldehyde. Infiltration began at the aortic side of the ductus and proceeded towards the pulmonary artery. The animals were allowed to recover from the operation and then studied at intervals from one to eight weeks following the procedure with hemodynamic measurements and radiographically to determine ductus patency.

RESULTS: A total of 11 animals underwent the procedure. Six had the ductus infiltrated with formalin and 5 with gluderaldehyde. The results are best summarized by the following table:

Number Patent/Total Number Animals

Time	Formalin	Gluteraldehyde
1 week	5/6	3/5
2 weeks	4/5	2/4
4 weeks	3/4	2/2
8 weeks	2/4	0/2

This data indicates that the method may be of use to maintain ductal patency. The major problem of this project was determining initial patency of the ductus. Further investigation into infiltrating the ductus of a fetal lamb in late gestation to assure initial patency is required. When initial patency can be adequately determined then long-term patency with infiltration can be compared.

This investigation will undergo further modification of the technique and further total analysis.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02676-01 SU

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Successful Repair of Traumatic Tricuspid Regurgitation 28 Years Following the Accident.

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

PI: Thomas J. Von Rueden, M.D., Clinical Associate, Clinic of Surgery, NHLBI

OTHER: Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Clinic of Surgery

SECTION

INSTITUTE AND LOCATION
National Heart, Lung and Blood Institute

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)

Traumatic isolated tricuspid regurgitation is rare. There are less than 50 reported cases. A case is described where successful repair has been performed 28 years later by prosthetic valve replacement. The literature is also reviewed.

765

PHS-6040
(Rev. 10-76)

DESCRIPTION: A case report and review of the literature concerning traumatic tricuspid regurgitation is presented. The case in point is a man who 28 years earlier was involved in a motorcycle accident. Within 9 days he was noted to have palpitations and a murmur. Subsequently he was diagnosed as having tricuspid regurgitation and paroxysmal atrial tachycardia. He was followed medically for 28 years and then sent here for evaluation. Tricuspid valve replacement with a porcine bioprosthesis was done. Postoperatively intracardiac pressures have returned to normal and the patient is now asymptomatic at 6 months. A review of the literature reveals that only one other reported case had a longer preoperative course before TVR was performed at 32 years.

PROPOSED COURSE: This case report is currently being written to be submitted for publication.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02677-01 SU

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Ventricular Function and Coronary Blood Flow in Chronic Ventricular Hypertrophy in Miniature Swine

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, NHIBI

OTHER: Steven R. Gundry, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Clinic of Surgery

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Pulmonary artery and infra-coronary aortic bands have been placed in young miniature swine weighing four to six Kg. Swine were chosen for the experimental animals because: 1) Growth allows for the gradual development of ventricular hypertrophy. 2) At maturity the animals weigh 50 to 60 Kg, being comparable in size to humans. 3) Swine do not have epicardial collateral coronary arteries as do dogs. Myocardial morphology, ventricular function and regional coronary blood flow will be investigated.

PHS-6040
(Rev. 10-76)

DESCRIPTION: Thirty swine have been thus far banded. Five of those died due to pulmonary infections. Seven of the animals with pulmonary artery bands have undergone late investigation. All had right ventricular systolic hypertension with PA pressures about 2/3 systemic. Four had compensated right ventricular hypertrophy and three had right ventricular failure as demonstrated by dilated right ventricles, enlarged livers and ascites.

Of the eight animals with infra-coronary aortic bands all have small outflow tract gradients; however, they had reached only one-half of their mature weights at the time of study.

In this model we plan to continue to study the development of ventricular hypertrophy and the relationships of hypertrophy to ventricular failure. The emphasis of future study will be placed upon the responses of the coronary circulation to right and left ventricular hypertrophy and failure.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02678-01 SU

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Regional Myocardial Blood Flow in Left Ventricular Hypertrophy

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
John H. Bell, M.D., Clinical Researcher-Clinic of Surgery, NHLBI

OTHERS: Joseph E. Pierce, DVM, Chief, Lab. Animal Medicine and Surgery Section, 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)
Laboratory of Animal Medicine and Surgery Section, NHLBI

LAB/BRANCH
Surgery

SECTION

INSTITUTE AND LOCATION
NHLBI-NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2-1/2	2-1/2	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Vulnerability of the subendocardium to ischemic injury has been documented during a variety of physiologic conditions in animals with normal hearts and those with left ventricular hypertrophy secondary to chronically elevated afterload distal to the coronary arteries. However, the lack of a laboratory model for left ventricular hypertrophy with aortic valve or subvalvular stenosis has restricted investigation of regional myocardial blood flow in this setting. To study this, we subjected Newfoundland dogs with congenital discrete subaortic stenosis and left ventricular hypertrophy to a variety of hemodynamic alterations while measuring regional myocardial blood flow with radioactive microspheres.

PHS-6040
(Rev. 10-76)

Description: To test the hypothesis that left ventricular hypertrophy (LVH) predisposes the subendocardium to ischemic injury, we studied regional myocardial blood flow using 9 ± 1μ radioactive microspheres in Newfoundland dogs with congenital discrete subaortic stenosis. Eleven dogs with LVH, left ventricle to body weight ratio (LV/BW) = 6.35 ± .46 Gm/Kg (mean ± SEM), were compared to 12 dogs without LVH/BW = 3.41 ± .12 Gm/Kg. The dogs were subjected to atrial pacing (221±4/min), ascending aortic constriction producing systolic hypertension (206 ± 5 mm.Hg), and creation of an aorto-right atrial fistula producing diastolic hypotension (38 ± 3 mm.Hg). LV endocardial to epicardial flow ratios were:

	NORMAL	LVH
CONTROL	.97 ± .04	.91 ± .05
PACED	.96 ± .04	.59 ± .07*
CONSTRICTION	.95 ± .04	.70 ± .06*
FISTULA	.86 ± .08	.57 ± .08+

* $p < .005$ and + $p < .02$ for LVH compared to normal

This study is the first one using a model for LVH with infra-coronary obstruction to demonstrate an increased subendocardial vulnerability to ischemic injury in the presence of LVH.

Proposed Course: This work has been submitted for presentation at the Scientific Sessions at the American Heart Association.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02679-01 SU

PERIOD COVERED October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Comparison of Regional Myocardial Blood Flow in Dogs with Left Ventricular Hypertrophy Secondary to Supra-aortic and Subaortic Outflow Tract Obstruction. Effects of a Critical Coronary Artery Stenosis.

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

PI: John Bell, M.D., Guest Worker, Clinic of Surgery, NHLBI
Michael Borkon, M.D., Clinical Associate, Clinic of Surgery, NHLBI

OTHERS: Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI
Andrew G. Morrow, M.D., Chief, Clinic of Surgery, NHLBI
Joseph E. Pierce, DVM, Chief, Lab. Animal Medicine and Surgery, NHLBI

COOPERATING UNITS (if any)

Laboratory of Animal Medicine and Surgery Section, NHLBI

LAB/BRANCH

Surgery

SECTION

INSTITUTE AND LOCATION

NHLBI-NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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)

Increased susceptibility of the left ventricular subendocardium to ischemic injury has been demonstrated during a variety of physiologic maneuvers in both normal and hypertrophied hearts. Other studies of regional myocardial blood flow in hearts with a critical coronary artery stenosis has documented even greater degrees of subendocardial ischemia distal to the stenosis. Studies were conducted on hearts with left ventricular hypertrophy (LVH) secondary to chronically elevated afterload distal to the coronary ostia. The lack of a laboratory model of LVH secondary to aortic valve or subvalve stenosis has hindered complete investigation of regional myocardial blood flow in these settings. Subsequently, no cooperative study has been reported regarding regional myocardial blood flow in hearts with LVH secondary to supra-aortic versus subaortic valve outflow obstruction. To further elucidate this, we are subjecting foxhound dogs with LVH secondary to ascending aortic constriction and Newfoundland dogs with LVH secondary to congenital discrete subaortic stenosis to several physiologic maneuvers after placing a critical stenosis on one major coronary artery. Regional myocardial blood flow is then measured using 9 ± micron μ radioactive microspheres.

771

Description: Foxhound puppies weighing between four and eight kilograms are anesthetized with halothane and ventilated via a Harvard respirator. Through a right thoracotomy, the ascending aorta is exposed. Approximately one and one-half centimeters above the aortic valve, a circumferential piece of quarter inch umbilical tape is placed such that after sutured in position there is a 25 to 30 mm. Hg gradient. The thoracotomy is closed in layers, and the animal is followed with serial ECG and chest roentgenographs for evidence of LVH. Only those animals with physical, electrocardiographic, or roentgenographic evidence of LVH are deemed acceptable for the experimental study.

Experimental dogs are then anesthetized with Chloralose (80 mg./Kg) and morphine sulfate (2 mg/Kg) and ventilated using a Harvard respirator. Through a left thoracotomy, catheters are placed to monitor and record left ventricular pressure, left atrial pressure, and systemic pressure, and the first derivative of left ventricular pressure. Coronary blood flow is measured by placing a Statham flow probe around the proximal left anterior descending artery (LAD). The cardiac output is monitored via a flow probe around the main pulmonary artery. Surface and endocardial ECG electrodes are placed and monitored throughout the experiment. The sodium and potassium levels and acid/base balance are monitored and kept physiologic. Regional myocardial blood flow is measured using 9-1 micron radioactive microspheres injected into the left atrium while withdrawing reference blood flow samples in the carotid artery. Coronary reactive hyperemia is measured after a ten second occlusion of the left anterior descending artery.

These parameters are recorded during each of the following periods:

(1) Control - period after dissection of coronary artery but before the screw constrictor is placed on the LAD.

(2) Baseline - after the "critical" coronary artery stenosis is placd on the LAD. Critical is defined as the abolition or reduction of reactive hyperemia to less than 10% of control.

(3) Rapid Atrial Pacing - bipolar right atrial pacing to 50% above control heart rate, or until aortic systolic pressure falls to 90 mm. Hg.

(4) Ascending aorta Banded - increased left ventricular afterload to 100% above control or until there is mechanical alternans.

At the completion of the banded maneuver, the LAD will be injected with methylene blue to determine myocardial flow distribution distal to the coronary stenosis. The animal is then sacrificed using potassium chloride, and the heart is fixed with 10% buffered formalin. The hearts are then sectioned for microsphere counting and pathological analysis. The distribution of myocardial blood flow within the area of critical stenosis will be analyzed and compared to the flow within area of the non-stenotic circumflex coronary artery. The flows of these two areas will then be compared between the two different groups of LVH dogs.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZC1 HL 02680-01 SU

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Regional Myocardial Blood Flow in Dogs with Left ventricular Hypertrophy Secondary to Subaortic Outflow Tract Obstruction; Effects of a Critical Coronary Artery Stenosis.

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

PI: John H. Bell, M.D., Guest Worker, Clinic of Surgery, NHLBI
A. Michael Borkon, M.D., Clinical Associate, Clinic of Surgery, NHLBI

OTHER: Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI
Andrew G. Morrow, M.D., Chief, Clinic of Surgery, NHLBI
Joseph E. Pierce, DVM, Chief, Lab Animal Medicine & Surgery Sec., NHLBI

COOPERATING UNITS (if any)
Lab Animal Medicine & Surgery Section, NHLBI

LAB/BRANCH
Clinic of Surgery

SECTION

INSTITUTE AND LOCATION
National Heart, Lung & Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	3	

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)

Increased susceptibility of the left ventricular subendocardium to ischemic injury has been demonstrated during a variety of physiologic maneuvers in both normal and hypertrophied hearts. Further studies of regional myocardial blood flow in hearts with left ventricular hypertrophy (LVH) and critical coronary artery stenosis has documented even greater degrees of ischemia. These studies were conducted on hearts with LVH secondary to chronically elevated ventricular afterload distal to the coronary ostia. The lack of a laboratory model of LVH secondary to aortic valve or subvalve stenosis has hindered complete investigation of regional myocardial blood flow in these settings. To elucidate this further, we are subjecting Newfoundland dogs with LV secondary to congenital discrete subaortic stenosis to different physiologic maneuvers after placing a critical stenosis on one major coronary artery. Regional myocardial blood flow is then measured using 9 ± 1 micron (μ) radioactive microspheres.

PHS-6040
(Rev. 10-76)

DESCRIPTION: Newfoundland dogs are anesthetized with Chloralose (80 mg/Kg) and Morphine Sulfate (2 mg/Kg) and ventilated using a Harvard respirator. Through a left thoracotomy, catheters are placed to monitor and record left ventricular pressure, left atrial pressure, systemic pressure, and the first derivative of left ventricular pressure. Coronary blood flow is measured by placing a Statham flow probe around the proximal left anterior descending artery (LAD). The cardiac output is monitored via a flow probe around the main pulmonary artery. Surface and endocardial ECG electrodes are placed and monitored throughout the experiment. Serum sodium and potassium levels and acid/base balance are monitored and kept physiologic. Regional myocardial blood flow is measured using 9-1 micron radioactive microspheres injected into the left atrium while withdrawing reference blood flow samples via the carotid artery. Coronary reactive hyperemia is measured after a ten second occlusion of the left anterior descending artery.

These parameters are recorded during each of the following periods:

1) Control - period after dissection of coronary artery but before the screw constrictor is placed on the LAD.

2) Baseline - after the 'critical' coronary artery stenosis is placed on the LAD. Critical is defined as the abolition or reduction of reactive hyperemia to less than 10% of control.

3) Rapid atrial pacing - bipolar right atrial pacing to 50% above control heart rate, or until aortic systolic pressure falls to 90 mmHg.

4) Ascending aorta banded - increased left ventricular afterload to 100% above control or until there is mechanical alternans.

At the completion of the banded maneuver, the LAD will be injected with methylene blue to determine myocardial flow distribution distal to the coronary stenosis. The animal is then sacrificed using potassium chloride, and the heart is fixed with 10% buffered formalin for 36 hours. The hearts are then sectioned for microsphere counting and pathological analysis. The distribution of regional myocardial blood flow within the area of critical stenosis will be analyzed and compared to the zone of distribution from the nonstenotic circumflex coronary artery.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02681-01 SU

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
LDH Isoenzymes and Evaluation of Myocardial Injury in the Period Immediately Following Cardiopulmonary Bypass (Application of a New Technique in Isoenzyme Analysis).

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

PI: Richard E. Michalik, M.D., Clinical Associate, Clinic of Surgery, NHLBI

OTHER: Nicholas Papadopoulos, PhD. Senior Staff, Clinical Pathology, CC

COOPERATING UNITS (if any)
Clinical Pathology Department, The Clinical Center

LAB/BRANCH
Clinic of Surgery

SECTION

INSTITUTE AND LOCATION
National Heart, Lung & Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3/4	3/4	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

In an attempt to further the ability to recognize accurately myocardial injury, an improved technique of LDH isoenzyme analysis is employed in serial sample analysis. It is hoped that close correlation of enzyme levels and the patient's clinical picture (including operative method) will show the new technique to be quite sensitive and quite specific.

Enzyme levels done by the new assay will be correlated with EKG data and similar comparisons will be made with EKG information and isoenzyme data obtained using the standard techniques available.

Blood samples will be obtained pre, intra- and postoperatively (the latter at regular intervals). Samples may be taken on a daily basis until discharge in those patients whose clinical course or electrocardiograms suggest injury.

DESCRIPTION: The use of LDH isoenzyme analysis in evaluation of myocardial infarct in a nonoperative setting is well established. However, hemolysis has, in the past, prevented application of previously available LDH isoenzyme analytical methods to the detection of myocardial injury in patients who have undergone cardiopulmonary bypass. A technique has been developed which does not permit changes in LDH-1, LDH-2 fractions due to hemolysis to obscure LDH changes due to myocardial infarction.

For purposes of the study patients of several select types will be analyzed:
1) Those having ventriculotomy performed (other than for aneurysmal changes).
2) Coronary artery bypass patients.
3) IHSS patients.

These were chosen for several reasons. The ventriculotomy patients have a known amount of injury to the myocardium, including a suture line in ventricular myocardium. Coronary bypass patients, particularly those not vented through the apex of the LV, have no direct myocardial injury, and IHSS patients have resection of ventricular myocardium but no myocardial suture line.

For purposes of the study, blood samples will be obtained preoperatively, intraoperatively before recirculation and 1, 4, 8, 16, 24, 48 hours postoperatively. Should any abnormality in either EKG or clinical course, or in the isoenzymes values appear, collection may be extended on a once daily schedule.

Copies of EKG tracing and clinical summary will be kept for analysis against enzyme values. It is hoped that with time enough, like cases could be accumulated of a certain type, that use of cardioplegia may be examined for its effectiveness in preservation, with patients who have had identical operations without cardioplegia serving as controls.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02682-01 SU

PERIOD COVERED October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Change in Canine Systemic-Pulmonary Shunt Flow by Afterload Reduction

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

PI: John S. Pfeifer, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Richard E. Michalik, M.D., Clinical Associate, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Surgery Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung and Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	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)

A model for the study of the feasibility of nonoperative, pharmacologic manipulation of the flow in a systemic-to-pulmonary shunt is produced by constructing a right subclavian-to-pulmonary shunt in acutely prepared canines. The appropriate measurements of flows and pressures are recorded so that total systemic, and pulmonary resistances might be calculated simultaneously with recorded shunt flows. Such baseline data are recorded after volume infusion has been employed to attain slightly elevated filling pressures in the heart. Afterload reduction is then instituted by sodium notroprusside infusion and increased incrementally while pressures and flows are constantly recorded, until the increases in afterload reduction are continued until arterial pressures fall below acceptable limits or until significant reduction in shunt flow is noted.

PHS-6040
(Rev. 10-76)

DESCRIPTION: At the present, the management of severe congestive heart failure in patients with existant systemic pulmonary shunts is limited to digitalization, diuresis, and operative intervention to modify the shunt. Most such patients are already digitalized and results of diuresis are usually less than hoped for, especially since such volume loss may result in diminished peripheral perfusion. Afterload reduction has been shown experimentally to cause significant decreases in intracardiac left to right shunts but its potential in reducing flow in a systemic-pulmonary shunt has not been explored.

In the acute setting, foxhounds undergo median sternotomy and creation of systemic (subclavian) to right pulmonary artery shunt with an interposed segment of synthetic graft material (for ease of preparation). Flow probes are then placed on the ascending and descending aorta, main pulmonary artery, and on the remaining portion of the subclavian artery proximal to the interposed synthetic graft. Material pressure monitoring is established for the PA, RA, LA, LV, proximal (ascending) aorta and the distal thoracic aorta. Once a steady state has been established infusion of sodium nitroprusside is begun at 1 mg/Kg/min, providing the left atrial mean pressure is 8-10 mm Hg. Should it be less than that value, volume infusion using whole blood and crystalloid is administered. The nitroprusside infusion is continued at the initial level for 10 minutes during which continuous recordings are made of the above listed pressures and flows. If no significant reduction in shunt flow or arterial pressure is seen, the infusion rate is increased by 1 mg/Kg/min every 10 minutes until either a 25% reduction in flow is noted, arterial mean pressure falls to or below 75% baseline or until an infusion rate of 10 mg/Kg/min is reached.

Once satisfactory reduction in shunt flow is reached, an attempt will be made to maintain that state for 90 minutes.

PROPOSED COURSE: Continue through 10-15 animals - then consider possibility of employing other agents to decrease shunt flow, including prostaglandins and ganglionic blocking agents.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02683-01 SU

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Evaluation of Polytetrafluorethylene (PTFE) Aorto-Coronary Grafts in Dogs

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

PI: Richard E. Michalik, M.D., Clinical Associate, Clinic of Surgery, NHLBI

OTHER: Michael Jones, Senior Surgeon, Clinic of Surgery, NHLBI
Daniel M. Goldfaden, Clinical Associate, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Clinic of Surgery

SECTION

INSTITUTE AND LOCATION
National Heart, Lung & Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1- 1/4	1 - 1/4	

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)

Primary purpose of this study is to evaluate PTFE grafts as a substitute for saphenous vein in bypass grafting for coronary artery disease. With the aid of cardiopulmonary bypass procedures several groups of dogs will be generated:

1) Saphenous vein graft bypasses to LAD. 2) Impra (a PTFE) grafts to LAD.
3) Possibly - Gore-tex grafts to LAD.

Subsequently all animals will undergo cardiac catheterization at regular intervals of 2-3 months, 8 months and 1 year postoperatively. A small number of animals from each arm of the study will be sacrificed following catheterization at each interval for gross and microscopic evaluation of the grafts.

779

DESCRIPTION: At this time no acceptable synthetic material is available for aorto-coronary bypass grafting. Of those materials thus far in use only "Gore-tex" a PTFE compound has had any success, and this has been limited. There are several problems inherent in the design/structure of Gore-tex that detract from its ease of use and perhaps are prohibitive to long-term patency of small caliber grafts. Impra, a more advanced design PTFE with more consistent mocrostructure, should be an improvement. A graphite lined Impra graft has been made available to us which should result in improvement of early patency rates.

There will be three groups of dogs created with approximately thirty (30) animals in each group:

1) Saphenous vein aorto-coronary (LAD) bypass graft with ligation LAD proximal to graft-coronary anastomosis.

2) Impra bypass graft.

3) Gore-tex.

The animals will be maintained and each animal will undergo catheterization for evaluation of graft patency at approximately 3 months. Two animals from each group (whose grafts have been determined to be patent by angio) will be sacrificed so that both gross and microscopic analyses of the grafts may be conducted. Re-evaluation will be carried out at 6 and 12 months.

Placement of the aorto-coronary grafts will require that each animal undergo median sternotomy and cardiopulmonary bypass with hypothermia and cardioplegia.

PROPOSED COURSE: Impra grafts are on hand, Gore-tex is available commerically, the literature has been reviewed with the possible exception of as yet undetermined articles concerning Gore-tex with reference to its use in the aorto-coronary position.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02684-01 SU

PERIOD COVERED
October 1, 1978 through September 30, 1979

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, NHLBI
Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Surgery

SECTION

INSTITUTE AND LOCATION
NHLBI-NIH, Bethesda, MD. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3/4	3/4	

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)

Despite advances in intraaortic 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.

781

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 ultrasonograpny, 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 balbon deflates, and blood drains from the coronary vein into the right atrium in the normal fashion. Myocardial dynamics are reassessed during this procedure. The procedure is then carried out to a variety of time endpoints, the animal is sacrificed and the myocardium studied.

This past year has been spent in design and modification of the pulsatile assist device and the balloon tipped catheter. Studies to date indicate that up to one hour after total LAD occlusion, dyskinetic and ischemic myocardium can return to normal function deriving perfusion only via coronary vein retroperfusion. Additionally, the massive intramyocardial hemorrhage seen with arterial-coronary venous anastomosis seems to be reduced since blood drains normally during systole with our model.

Proposed Couse of Project: Full experimental procedural testing will commence this summer employing a variety of ischemic time periods and total pumping times in an effort to determine this method's potential clinical applications. If confirmed, this device offers a transvenous approach to temporarily assist the failing ischemic myocardium.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02685-01 SU

PERIOD COVERED October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Sublingual Nitroglycerin: Effects on arterial oxygen tension

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: Kenneth Kent, M.D., Chief, Cardiovascular Diagnosis Section, Cardiology
Michael Jones, M.D., Senior Surgeon, Clinic of Surgery

COOPERATING UNITS (if any)

Cardiology Branch, NHLBI, Nuclear Medicine, Clinical Center

LAB/BRANCH

Clinic of Surgery

SECTION

INSTITUTE AND LOCATION

National Heart, Lung & Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/2	1/2	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A recent published report linked the administration of sublingual nitroglycerin with subsequent arterial hypoxemia in patients undergoing coronary artery bypass grafting. We have designed a protocol to investigate the following: 1) to determine whether sublingually administered nitroglycerin causes a decrease in arterial oxygen tension; 2) to determine the degree of arterial hypoxemia sublingual nitroglycerin may cause in patients with coronary artery disease; 3) to investigate the mechanism of action, if sublingual nitroglycerin does cause a reduction in arterial oxygen tension.

783

PHS-6040
(Rev. 10-76)

DESCRIPTION: Patients admitted to the Cardiology and Cardiac Surgery Services for coronary artery bypass grafting are evaluated with the following studies: 1) Resting arterial blood gases on room air are drawn, after which 0.6 mg. NTG is administered sublingually. After 10 minutes, a second arterial blood gas is drawn -- pH, pO_2, pCO_2, O_2 saturation, hemoglobin and methemoglobin are measured. 2) All patients who come to operation have placement of a thermodilution Swan Ganz catheter. Postoperatively, following a one hour stabilization on 100% FiO_2, the following parameters are measured or derived in a resting state and 10 minutes following 0.6 mg. administration of sublingual nitroglycerin: pO_2, pCO_2, pH, O_2 saturation, hemoglobin, methemoglobin, heart rate, EKG, systolic arterial pressure, diastolic and mean arterial pressure, cardiac output and index, systemic vascular resistance, pulmonary vascular resistance and left and right ventricular stroke work. 3) In future patients, prior to operation, ventilation/perfusion scans will be performed in nuclear medicine, in a resting state and 10 minutes following sublingual nitroglycerin administration.

RESULTS: At the present time, 6 patients with coronary artery disease have been studied using the above methods. Four of the patients had significant drops in arterial oxygen tension following sublingual nitroglycerin, while two had little or no change. As the series continues we hope to be able to identify the cause of this hypoxemia by studying nitroglycerin's effect on ventilation/perfusion mismatch in the lung. If this research identifies nitroglycerin as a potentially causitive agent for arterial hypoxemia in certain patients, then its routine use in antianginal therapy might be modified and those patients at risk identified.

COURSE: The present study will be continued until a suitable number of subjects has been evaluated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02686-01 SU

PERIOD COVERED
October 1, 1978 through September 30, 1979

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

OTHER: Victor Ferrans, M.D., PhD., Chief, Ultrastructure Sec., Pathology Br.
Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, 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:
3/4	3/4	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Endothelial changes in saphenous veins harvested for coronary artery bypass grafts were found when veins prepared in this manner were examined using the scanning and transmission electron microscopes. Using current distention pressures and preservation techniques, severe endothelial disruption was found. As the project progresses, we will compare 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. 10-76)

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. Early results indicate that significant and irreversible damage occurs to the endothelium of vein grafts during normal handling and distention. These changes, in vivo, might cause platelet aggregation, intimal hyperplasia or early atherosclerotic lesions.

PROPOSED COURSE: Final comparison is now being made between current vein handling techniques and normal venous architecture. We will then examine other methods of vein handling and preservation techniques on the alteration of endothelium, to arrive at recommendations for the most effective method of vein harvesting and handling during coronary artery bypass graft procedures.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02687-01 SU

PERIOD COVERED

October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Unidirectional Intra-atrial Flap Valve: Evaluation in Experimental Pulmonary Hypertension and Right Ventricular Failure

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., 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

COOPERATING UNITS (if any)

LAB/BRANCH

Surgery

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

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)

Surgical repair of intracardiac defects with pulmonary hypertension is still associated with a 20 to 30 percent mortality, often secondary to right ventricular dilatation and failure. Unloading of the right ventricle may be accomplished by venous unloading only at the expense of left ventricular output. Therefore we are evaluating a unidirectional intra-atrial flap valve that allows right-to-left shunts when right ventricular filling pressures are elevated, thus decompressing the right ventricle while maintaining or improving left ventricular output.

787

Description: Experimental animals are given a pressure load to the right ventricle by means of an adjustable band around the pulmonary artery and physiologic parameters are measured over a variety of resistances to right ventricular outflow. Using extracorporeal circulation, a unidirectional flap valve of dacron reinforced Lycra is implanted in the atrial septum which allows a right-to-left shunt only when right atrial pressures exceed left atrial pressures. Under normal conditions, the valve remains closed, but during increasing right ventricular dilatation and obstruction, right atrial pressure exceeds left atrial pressure and shunts blood right-to-left, thus acting as a "pop-off valve." In initial design studies, right-to-left shunting occurred when right ventricular pressures in dogs exceeded 50 mm. Hg, and arterial hypoxemia was within tolerable limits. Cardiac output in these dogs was maintained and right ventricular dilatation did not result. Similar pressures in control animals caused right ventricular dilatation and numerous arrhythmias.

Proposed Course: This past year has been spent in design and fabrication of an anti-thrombogenic valve which can be implantable but which can become tissue covered with time. After encouraging initial testing, we plan testing the valve in acute studies to determine its potential for development. This will be followed by chronic implantation experiments.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02688-01 SU

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
The Effects of Dobutamine and Dopamine on Patients with Pulmonary Hypertension.

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

PI: Thomas J. Von Rueden, M.D., Clinical Associate, Clinic of Surgery, NHLBI

OTHER: Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Surgery

SECTION

INSTITUTE AND LOCATION
NHLBI-NIH, Bethesda, MD 20205

TOTAL MANYEARS: 3/4	PROFESSIONAL: 3/4	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)

Patients with pulmonary hypertension are a heterogenous group with respect to the etiology of the pulmonary vascular changes. The pulmonary vascular resistance is variable in its capacity to respond to stimuli. In general these patients have a difficult postoperative course from both a respiratory and cardiac viewpoint. Dobutamine and dopamine are the two main beta stimulators for low output postoperatively. Their effect on the pulmonary vascular system has not been well studied. We have studied a group of postoperative cardiac surgical patients with preoperative pulmonary hypersion. Dose-response curves for both dopamine and dobutamine were developed. Hemodynamic parameters were recorded and from these the systemic and pulmonary vascular resistances were calculated. While both drugs significantly lower systemic vascular resistance, only dopamine significantly reduces pulmonary resistance. We conclude that the use of dopamine in these patients can be advantageous in their postoperative management.

789

Description: Ten patients with preoperative pulmonary hypertension have been evaluated. We have defined pulmonary hypertension as being a mean pulmonary artery pressure of 40 mm. or greater. The primary cardiac lesions are congenital and acquired; the majority of patients have rheumatic mitral valvular disease. All operations are performed using a midline sternotomy. Cardiopulmonary bypass is utilized with moderate hypothermia. Myocardial preservation is achieved using topical hypothermia and cold crystalloid cardioplegia. Before discontinuation of cardiopulmonary bypass, a left atrial pressure line and a triple-lumen Swan-Ganz catheter are placed. The study is commenced only when the patient's clinical course has stabilized. This is usually the morning following operation. Dose response curves with both dobutamine and dopamine are developed. Initially dopamine is infused through a central venous line at a rate of 2 mcg/Kg/min. At 15 minute intervals the dosage is increased by 2 mcg/Kg/min until the mean aortic pressure goes up 15 mm. or the heart rate increased by 30. At each interval the following parameters are recorded : BP, MAP, HR, MRA, PAS, PAD, PAM, LA, PCW, CO. From these the CI, PVR and SVR are calculated. Preliminary data show that both drugs are equally effective in raising the cardiac index and decreasing the systemic vascular resistance. However, only dopamine significantly lowers the pulmonary vascular resistance. Pulmonary artery pressures are increased by both drugs but this can be explained by the concomitant increase in flow.

Proposed Course: This work is in the process of being written for submission to Circulation for publication.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02689-01 SU

PERIOD COVERED

October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Successful Correction of Tricuspid Valvular Insufficiency Twenty-eight Years Following Trauma.

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

PI: Thomas J. Von Rueden, M.D., Clinical Associate, Clinic of Surgery, NHLBI

OTHER: Michael Borkon, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

SURGERY

SECTION

INSTITUTE AND LOCATION

NHLBI-NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/2	1/2	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Traumatically induced lesions of the cardiac valves affects the aortic valve most commonly, then the mitral, and rarely the tricuspid. Valvular injury is almost invariably associated with other severe cardiac injury, except the aortic valve which is often injured alone. Damage to the tricuspid valve typically results in valvular insufficiency due to chordal rupture or papillary muscle operation. Hemodynamically, patients usually tolerate traumatically induced tricuspid insufficiency very well as compared to similar lesions of the aortic and mitral valves; patients often go 10-20 years before requiring medical intervention. In addition, prosthetic valve replacement can provide excellent results even in these patients with long standing disease. We shall present a patient with isolated tricuspid valve insufficiency resulting from a motorcycle accident who subsequently twenty-eight years later underwent tricuspid valve replacement with excellent results.

PHS-6040
(Rev. 10-76)

Description: A case report is presented of a 50 year old man from India who developed tricuspid valvular insufficiency due to trauma. He was followed as an outpatient in India and remained functional Class II for 27 years. He was referred to the National Heart Institute when he decompensated to functional Class IV. He underwent cardiac catheterization which showed severe tricuspid valvular incompetence. His right atrial pressure was markedly elevated with V waves of 28 mm. Hg. He was brought to the operating room where his tricuspid valve was found to have torn chordae tendinae such that the entire anterior leaflet was flail. The valve was replaced with a #35 Hancock bioprosthesis. His postoperative course was uneventful. Six months later he is back to work and in functional Class II. Cardiac catheterization now shows marked improvement with mean right atrial pressures of 7 mm. Hg. A review of the literature shows only 16 such cases of isolated tricuspid valve regurgitation from trauma. As in our patient, the traumatically induced tricuspid valvular incompetence is well tolerated; patients often go 15-20 years before requiring medical intervention. Prosthetic valve replacement provides excellent results even in patients with long standing disease.

Proposed Course: This work is in manuscript form and will be submitted as a case report and review of the literature.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02690-01 SU

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Late Results Following Operative Correction of Congenital Aortic Valvular Stenosis.

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

PI: Thomas J. Von Rueden, M.D., Clinical Associate, Clinic of Surgery, NHLBI

OTHER: Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI
Andrew G. Morrow, M.D., Chief, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH Surgery

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Congenital aortic stenosis comprises 3-5% of all congenital heart disease. Left ventricular obstruction at the valvular level is the most common form. We have reviewed 47 patients who underwent aortic valvotomy for congenital valvular aortic stenosis. These patients have been followed 5-21 years, mean followup is 13 years. Although 2/3 of the patients are asymptomatic, late valve related complications mandate continued patient observation.

793

PHS-6040
(Rev. 10-76)

Description: Between 1958 and 1974, 47 patients, ages 1-31 years, with congenital aortic valvular stenosis underwent aortic valvotomy; there were no operative deaths. At the time of operation 45 patients had bicuspid valves, and 2 patients had unicuspid valves; 3 patients had hypoplastic outflow tracts. Forty-five patients have been followed postoperatively for 5-21 years (mean 13 years). Currently 6% (30/45) are asymptomatic. Aortic regurgitation is evident in 22 patients, but in 14 of them the diastolic murmur is only grade I-II/VI. Postoperative cardiac catheterization has been performed in 38 patients. The average peak systolic valve gradient was 30 mm. Hg; the average aortic valve index (17 patients) was 0.66 cm^2/M^2. Seven of 45 patients (16%) have required aortic valve replacement from 5-17 years (mean 11 years) postoperatively. Three of 45 patients (17%) required treatment of bacterial endocarditis. Aortic valvotomy provides satisfactory palliation for patients with congenital valvular aortic stenosis, but late valve-related complications mandate continuing patient observation.

Proposed Course: This abstract has been sent to the American Heart Association Meeting.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02691-01 SU

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
A Report of Enflurane - Induced Hepatitis.

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

PI: Harry Michael Lewis, M.D., Clinical Associate, Clinic of Surgery, NHLBI

OTHERS: A. Michael Borkon, M.D., Clinical Associate, Clinic of Surg., NHLBI
William M. Dixon, M.D., Anesthesiologist, Clinical Center, NIH
Charles L. McIntosh, M.D., Ph.D., Senior Surgeon, Clinic of Surg. NHLBI

COOPERATING UNITS (if any)
·Department of Anesthesiology

LAB/BRANCH
Surgery

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	4	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A young white male underwent open heart surgery for aortic valve replacement and developed acute, postoperative hepatitis. A detailed examination of prior toxic agent exposure as well as a review of medications used in a postoperative period suggest the possibility of enflurane-induced hepatitis.

795

PHS-6040
(Rev. 10-76)

Description: A 29 year old white male was admitted for aortic valve replacement for aortic insufficiency following acute episodes of acute rheumatic fever at 9 and 10 years of age. He was first shown to have 4+ aortic insufficiency in May 1972 but was followed until February 1978 when he was recatheterized. At that time he was noted to have decreased left ventricular function and plans were made for aortic valve replacement. On April 13, 1978 he underwent #27 porcine aortic valve replacement. His pump time was 2 hours 7 minutes with minimum temperature of 28.5 centigrade. Over the next several days his bilirubin climbed to a peak of 13.0 with direct bilirubin of 12.0, SGPT peaked at 1,260, SGOT peaked at 504, alkaline phosphatase peaked at 463. His current drug history was examined and he was noted to have received dopamine, lidocaine, cefazolin, acetaminophen, quinidine, furosemide, digoxin, morphine, $FeSO_4$, folic acid, and ethchlorvynol as well as enflurane. By time of discharge, May 19, 1978, his bilirubin was 3.2 with direct bilirubin of 2.8. SGPT was 27, SGOT was 41, alkaline phosphatase was 84. His past medical history was positive for multiple transfusions. In addition, his occupational exposure was not clearly defined but it is of note that he lived in a town with many chemical plants. At the time of his followup catheterization, his bilirubin was 0.5 with a direct of 0.1. His SGPT and SGOT were still elevated at 140 and 75 respectively. The acute onset of his hepatitis suggests that it was chemically induced by a drug which in this case might be enflurane.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02692-01 SU

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Adaptation of a General Medical Information System to a Postcardiac Surgery ICU

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

PI: Harry Michael Lewis, M.D., Clinical Associate, Clinic of Surgery, NHLBI

OTHER: Lily Ng, R.N., Head Nurse, 6-West, Department of Nursing, C.C.
Thomas L. Lewis, M.D., Chief, Office of Clinical & Management Systems

COOPERATING UNITS (if any)
Department of Nursing & Office of Clinical & Management Systems, CC

LAB/BRANCH
Clinic of Surgery

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, & Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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)

A Technicon Medical Information System was instituted in the Clinical Center, NIH, to provide accurate data collection, retrieval, and transmission of medical orders for the general patient population of NIH. By the use of specially designed software, this system was adapted for use in an intensive care unit.

PHS-6040
(Rev. 10-76)

DESCRIPTION: The Technicon Medical Information System is a general patient care medical information system which has been in use in the Clinical Center, NIH. This system provides easy access to the NIH patient population in general but more especially to those actively hospitalized. In-patient data include standard admission data such as name, age, address, date of birth, diagnosis, and referring physician. It also includes complete records of radiology requests and results, pharmacy, respiratory therapy, and nursing orders as well as appropriate charting against these orders, and transmission of orders for other services such as laboratory requests. Because of the generality of the system it was initially deemed too cumbersome to install in an intensive care unit where there would be a large volume of medical orders and nursing charting entries. This system was streamlined by separating order categories into routine and occasional use groups. Routine categories were placed on a Master Order Guide with the rest being placed on a Departmental Order Guide. Each category on both guides was examined in detail and the same process performed. Routines in each category were identified and organized into order pathways which permitted rapid access while the occasional orders in each category were placed in groups which had much slower access times. As a result the usual and frequent orders given in an ICU setting could be accurately and rapidly written with all the side benefits of the system accruing such as interaction with the pharmacy for supplies and daily and weekly dose charting, etc. while the flexibility of the general system was retained.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02693-01 SU

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Adaptation of a Programmable Scientific Calculator to a Post-Cardiac Surgery Intensive Care Unit.

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

PI: Harry Michael Lewis, M.D., Clinical Associate, Clinic of Surgery, NHLBI

OTHER: A. Michael Borkon, M.D., Clinical Associate, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Surgery

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 2	PROFESSIONAL: 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)

A Texas Instruments TI-59 Programmable Scientific Calculator was programmed to make available to physicians and nurses frequently used patient care mathematical formulas in the post cardiac surgery ICU.

799

PHS-6040
(Rev. 10-76)

Description: A Texas Instruments TI-59 Programmable Scientific Calculator and PC-100C Thermal Printer were programmed to create a device which would be self-instructional or at least require minimal assistance in the generation of patient care mathematical formulas. This was accomplished by devoting large blocks of the calculator's internal memory to alpha-numeric storage with the result that instructional headings would appear prior to each datum entry. These conversational programs allow the calculation of body surface area in m^2 from the patient's weight in kg and height in cm's, cardiac index from the patient's body surface area and cardiac output in l/min. Pulmonary and systemic vascular resistances in Wood units from cardiac output, pulmonary capillary wedge pressure, central venous pressure and systemic pressure, drug infusion rates in ml/hr. = gtt/min. from concentration in mg/ml and desired rates in ug/kg/min, and creatinine clearance in cc/min. from serum creatinine and 24 hour urine creatinine excretion.

Proposed Course: Development of the specific program is continuing at the present time. Ultimate development will include creation of a solid-state software package. However, manufacturing considerations require the creation of a minimum of 250 units. A description of the proposed software will be submitted for publication where appropriate.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02694-01 SU

PERIOD COVERED

October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

An Evaluation of Gorlin's Constant with Respect to Porcine Aortic Valve Replacement.

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

PI: Harry Michael Lewis, M.D., Clinical Associate, Clinic of Surgery, NHLBI

OTHER: A. Michael Borkon, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Charles L. McIntosh, M.D., Senior Surgeon, Clinic of Surgery, NHLBI
Andrew G. Morrow, M.D., Chief, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Surgery

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	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)

Gorlin's Constant was developed for the determination of valve area. It was derived from stenotic mitral valves and extrapolated for aortic valves. This study evaluates the constant in relation to porcine aortic valves which had their valve area determined prior to implantation.

801

Description: Gorlin's Constant was initially derived by the planimetry of stenotic mitral valves at autopsy. A formula for calculating valve area from hemodynamic determinations was subsequently developed and included a constant, K.This formula is valve area (cm^2) = per second valve flow (cc/sec) ÷ mean gradient (mm Hg) x K where per second valve flow = cardiac output (cc/min) ÷ mean gradient duration (sec) x heart rate (beats/min). This formula was then extrapolated to include aortic valve area determinations. Hemodynamic data were collected on 72 postoperative patients who had undergone porcine prosthetic aortic valve replacement for a variety of lesions. Of these 72 patients 50% had demonstrable gradients which allowed determination of prosthetic aortic valve area by Gorlin 's formula. This derived valve area was then compared with the valve area obtained by the manufacturer using orifice planimetry at the time of manufacture. The hemodynamic data suggested that the current Gorlin formula significantly underestimated actual valve area obtained by planimetry. Correction of Gorlin's formula can be made by adjusting the constant, K. To this end a conversational computer program for a DEC-10 computer system was written, and this provided on-line evaluation of the postoperative hemodynamic data solving the Gorlin formula for K. $K = (CO \times \sqrt{MG}) \div AVA \times MGD \times HR$. However, a letter from Hancock Laboratories on June 15, 1979 stated that an independent in-vitro estimation of valve orifice size substantiated the original Gorlin formula and constant. As a result, a correction of Gorlin's K is not in order.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02695-01 SU

PERIOD COVERED October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Evaluation of the Right ventricular Myocardium in Patients with Congenital Heart Disease: Morphological and Clinical Relationships

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
Victor J. Ferrans, M.D., PhD., Chief, Ultrastructure Section, Pathology

OTHER: William C. Roberts, M.D., Chief, Pathology Branch, NHLBI
Andrew G. Morrow, M.D., Chief, Clinic of Surgery, NHLBI

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:
7	7	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Clinical studies in patients with valvular heart disease and with congenital heart disease suggest that a "myocardial factor" related to dysfunction of the heart muscle itself is responsible for unsatisfactory postoperative clinical courses in certain patients. Additionally, controversies exist whether to perform palliative operations or anatomically corrective operations upon patients with congenital heart anomalies. The proper timing of operations is questioned as well. No information is available concerning the time courses of morphological changes of myocardial hypertrophy in humans and the relationships of morphological changes to postoperative clinical results.

Over the previous eight years we have compared light and electron microscopic observations with clinical courses of patients undergoing operations for congenital heart anomalies associated with right ventricular systolic pressure overload. We have published descriptions of myocardial morphological alterations of right ventricular hypertrophy and degeneration in these patients during previous years. During the past year we have summarized those observations and have included additional patients under morphological and long-term clinical follow-up study.

803

DESCRIPTION: Based upon our studies, we generalize that patients with congenital heart disease and right ventricular hypertrophy, who are over 15 to 20 years old, may experience a less than optimal postoperative clinical course.

At present we have studied by light and electron microscopy the myocardium obtained at operation from 75 patients with right ventricular pressure overload (tetralogy of Fallot physiology). The patients have been divided into four groups: I (36 patients) aged 10 months to 10 years; II (22 patients) aged 11 to 20 years; III (8 patients) aged 21 to 29 years; and, IV (9 patients) aged 30 to 53 years.

Half of group III and 78% of group IV patients suffered clinically apparent cardiac failure, major arrhythmias, peri-operative mortality, and/or late deaths. Alterations of cardiac hypertrophy and degeneration were severe in both groups of patients. Prominent interstitial fibrosis was observed in groups I, II, III, and IV at frequencies of 19%, 23%, 25%, and 100%, respectively. Myofibrillar lysis, myelin figures, smooth endoplasmic reticulum proliferation, and cell membrane associated spherical mocroparticles were less common in groups I, II, and III (0% to 25%) than in group IV (67% to 100%). Cellular atrophy, disorganization of cells and myofibrils, lipid accumulation, intracytoplasmic junctions, and thickened basal laminae were frequent in cardiac muscle from groups III and IV.

Thus the degenerative morphological atlerations of chronic right ventricular hypertrophy in man appeared related to clinical myocardial dysfunction. These relationships have implications for patients' long-term prognoses, the type and timing of their operations, and their requirements for intraoperative myocardial protection.

PROPOSED COURSE: 1) Tissues obtained from six additional patients in groups III and IV (over age 20 years) will be evaluated and compared with the patients' clinical courses. 2) We have right ventricular myocardial biopsies from 37 additional patients in groups I and II (age 3 days to 20 years). We will subdivide these patients by ages: 3 days to 5 years, 6 to 10 years, 11 to 15 years, and 16 to 20 years for study. Following this, the total number of patients in all groups, which we have studied will be 118. We anticipate that the information obtained from the study of all patients with right ventricular systolic overload will allow recommendations regarding the ideal time for palliative or anatomically corrective operations. 3) Approximately 70 patients are available for a five year followup study, which is planned in the next year. 4) A noninvasive evaluation of right ventricular function by two dimensional real time echocardiography and by radionuclide angiography is planned in cooperation with the Cardiology Branch. This evaluation of right ventricular function will be compared to our morphological evaluation of the patients' right ventricular myocardium.

PUBLICATIONS:

Jones, M., Ferrans, V.J.: Right ventricular hypertrophy: Clinical and ultrastructural relationships. Brit. Heart J. 40:457, 1978.

Jones, M., Ferrans, V.J.: Myocardial ultrastructure in children and adults with congenital heart disease. In Congenital Heart Disease in Adults (Roberts, W.C., ed). F.A. Davis, Philadelphia, 1979.

ANNUAL REPORT OF THE
LABORATORY OF TECHNICAL DEVELOPMENT
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
OCTOBER 1,1978 TO SEPTEMBER 30, 1979

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. Industrial instrumentation on the other hand tends to develop only ideas with potential sales appeal.

The Section on Biophysical Instrumentation is continuing to investigate the limits of enzyme kinetics, and binding by several mechanisms.

Advances in solid state material science suggest that smaller and faster temperature sensors could be constructed and applied to calorimetric measurements of enzymes in the relatively low concentrations limited by reaction rates and the demands of fast mixing reaction systems.

Special coatings for thermistors and new capacitor temperature sensors were developed here and by contract with the National Bureau of Standards. A time resolution of 3msec. at 10^{-4} degrees C sensitivity has been attained. Data handling and mathematical correction methods have been adapted and optimized to the limits of computer technology

Fast reactions and more subtle changes in molecular structure may be studied by the use of laser ultra fast picosecond = (10^{-12} Sec) spectroscopy and Mossbauer spectroscopy. Current efforts to evaluate these involve the construction of the laser system that can photolyze or excite molecular species to short lived states and then time resolve the specroscopic adsorpotion variations of the time course of the molecular conformations which occur while the molecules are recombining or relaxing to a new state. The system is performing satisfactory as stages of its development advance.

Mossbauer spectroscopy, largely limited to iron containing molecules, has a spectacular resolution capability to measure the small iron nuclear changes induced by electronic and conformational changes in the host molecule. These are useful in the study of the mechanism and action of the host molecule and its substrate. Some data already obtained has cast doubts on the energy exchange mechanism currently held for Ferrichrome A relaxation.

The development of these facilites provides opportunities for NIH scientists to evaluate these new approaches to molecular behavior.

This laboratory continues to study fluorescence and phosporesence instrumentation for application to problems such as membrane structure in relation to functional properties.

Fluorescence methods were used to study certain problems of concern in biomedical sciences. The areas investigated included: 1. The effect of Vitamin A analogs on the fluidity of cell membrane lipids in relationship to the toxicity of the analogs. 2. The binding of metal ions to tryptophan peptides, as determined by fluorescence quenching. 3. The functional integrity of phospholipid bilayer membranes, as affected by heating or treatment with phospholipases. The changes in the membrane structure were followed by a fluorescent dye leakage method. 4. The effects of salt and phospholipids on the activity of a number of enzymes such as alcohol dehydrogenases and glutamate dehydrogenase.

Separation techniques based on complex behavior of two phase liquid extraction phases in coiled tubes exposed to centrifugal forces continues to provide the basis for instrumental methods with special advantages.

A versatile horizontal flow-through coil planet centrifuge has been introduced for performing countercurrent chromatography. The apparatus carries a pair of coiled separation columns. Both of these allow continuous elution simultaneously without the use of rotating seals. One column enables preparative-scale separations and the other, analytical-scale separations both with a high partition efficiency comparable to that obtained in liquid chromatography but without the complications arising from the use of solid supports. Capability of the apparatus was demonstrated on separations of dinitrophyenyl (DNP) amino acids and peptides using typical two-phase solvent systems.

The Pulmonary and Cardiac Assist Devices Section has demonstrated that the removal of CO_2 via an extracorporeal membrane lung can obviate the necessity of to and fro gas flow in and out of the natural lungs. The blood simply removes oxygen from the alveolar gas and new oxygen flows in passively to replace consumed oxygen.

The CO_2 removal is accomplished by stripping several times the normal A-V difference per blood flow in the extracorporeal circuit so that only a fraction of total blood flow need be pumped thru the extracorporeal circuit. A normal arterial PCO_2 relieves the stimulus to respiration and minimal pressure or motion of the lung

presumably is more dersirable for healing perforations and reducing spread of infection.

Apneic respiration has been demonstrated in sheep and newborn pre-term lambs and proven to provide extended respiratory support with a fourth or less extracorporeal blood flow, no damage to normal lungs and no interference with maturation of pre-term lungs that would not have survived without respiratory support in ARF.

A human application of the system was reported from our Italian collaborators to have confirmed the capacity of the system to provide respiratory support in adult respiratory failure in a very sick individual.

The spiral coil oxygenator as modified above for CO_2 removal can also be modified to contain functioning cells that are nourished via a membrane permeable to nutrients and gases but not to cells. It has already been demonstrated that cells of foreign species can survive and grow in such a chamber when the other side of the membrane is continuously perfused by extracorporeal blood.

Surviving xenograft liver cells for example contained in the "oxygenator" could functionally be active via the separating membrane and nourished by the extracorporeal flow of a donor liver deficient patient.

Tissue and cell harvesting by means of modifications to flow thru centrifuges have been explored in a centrifuge system without rotating seals along similar lines that were used in our countercurrent separations methods. The system has reduced the sedimentation distance and induced backward flow to optimize the separations of formed elements in blood. Elimination of contamination into or out of blood and tissue suspension and faster gentler separations have been demonstrated.

In tissue culture systems for the study of cell transport for growth and function of cells in sheets grown on permeable membranes instead of glass or plastic surfaces has been studied with the aim of providing these cells with an opportunity to exchange nutrient and secretions from both sides of the sheet. Microporus membranes coated with collagen to provide for adhesion seems to provide the environment which permits nutrition, secretion and microscopic visualization as well as isolation of the membrane faces for bioelectrical measurements and control.

Microchemical methods applicable to biosystems where the sample source is limited or repeated sampling is required without depletion have been explored for gasses and specific ions of interest.

Excitation of emission spectra by electric discharges in gasses and chemiluminescent methods for nitrogen and ammonia had high sensitivity and linearity. The system remmains to be applied.

To avoid the depletion of oxygen at measuring PO_2 electrodes without reducing total polarographic current to noise levels or slowing the response time a membrane that presents the effect of an extended array of fast micro points in parallel was constructed. Material limitations have not been solved but a new silicone cement has promise.

Microcolorimetric systems demand a long light path length with minimal volume either for flow or discrete samples. Fiber optics, solid state detectors and sources using specific color complexes were explored for micromagnesium determination in submicroliter volumes. Absorption and reference data were handled by digital electronic system to provide a simple ratio response.

Tissue blood flow instrumentation has been significantly improved this year by conducting the light to and from the tissue via new graded index fiber optics, a solid state detector and improvements in the method of data processing the doppler backscatter spectrum. The flexability now permits easy access to patients or tissue. The new analysis resolves flow wave forms and increases sensitivity. Several studies in collaboration with other NIH laboratories are meauring muscle flow and skin flow in peripheral vasuclar disease.

Nuclear magnetic resonance apparatus using the new superconducting magnet in our contract program for intracerebral flow has now incorporated electronic systems developed and tested for peripheral vessel flow.

The high magnetic fields now available have demonstrated large signals in peripheral vessels as well as very large signals directly from the aorta. While these sites are accessable by other methods they demonstrate the power of the system to penetrate and receive signals entirely by magnetic and electromagnetic fields. Marking and detection of localized cerebral flow within the skull not accessable to ultrasound is anticipated but the short life of the magnetic marker in the blood remains a problem.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01404-11 LTD

PERIOD COVERED

October 1, 1978 to September 30, 1979

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

P.I. :	T. Kolobow	Chief, Pulmonary & Cardiac Assist Devices	LTD NHLBI
Others:	A. Pesenti	Visiting Fellow	LTD NHLBI
	L. Gattinoni		U. of Milan, Italy

COOPERATING UNITS (if any)

University of Milan, Instituto Anestesia E. Rianimazione

LAB/BRANCH

Laboratory of Technical Development

SECTION

Pulmonary and Cardiac Assist Devices

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1	1/2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The purpose of this project is to 1. develop improved membranes, membrane lungs, and membrane lung systems for prolonged pulmonary and cardiopulmonary support. Major efforts have centered on developing improved carbon dioxide membrane lungs for enhanced removal of carbon dioxide from extracorporeal blood. 2. to develop methods for the extracorporeal removal of carbon dioxide for the control of breathing. 3. to develop an animal model system using the pre-term lamb to study the use of the carbon dioxide membrane lung on the development of hyaline membrane disease (HMD).

BIC

Objectives:

1. To develop a membrane lung system for long term support.

The membrane lung is inherently more physiological than the widely used bubble oxygenator. Since the emergence of highly reliable and simple to use membrane lungs, there has been a progressively greater use of membrane lungs in open heart procedures, and the trend is continuing.

For long term applications, the membrane lung is a practical alternative for safe blood gas exchange. For these applications, the membrane lungs have been used for up to 3 weeks with little blood trauma. Bubble oxygenators have a practical limit of well under one day due to severe progressive hematological derangement.

2. To develop an artificial lung optimized for carbon dioxide removal.

The role of carbon dioxide is usually given little attention, as problems of carbon dioxide retention are relatively rare. However, when carbon dioxide retention does occur, it is an ominous sign in both acute and chronic respirtory failure.

We have been attracted by the role of carbon dioxide in mechanical pulmonary ventilation, and particularly in these patients who become severely ill of Acute Respiratory Failure (ARF), a majority of whom subsequently die. All the carbon dioxide produced at rest can be eliminated through an efficient extracorporeal membrane lung at a blood flow of only 500-1000 ml/min. By removing a major fraction of carbon dioxide produced by the body, one could modify spontaneous ventilation, as well as mechanical pulmonary ventilation.

It is known that prolonged mechanical pulmonary ventilation substantially damages lung performance in healthy animals, and in man, and similarly impairs the function of other internal organ systems such as the kidney and liver. It was hoped that multiorgan involvement could be reduced by reducing mechanical pulmonary ventilation by removing some of the carbon dioxide produced through an extracorporeal membrane lung.

Similarly, we expect that in chronic respiratory failure, it may be possible to lower total body carbon dioxide content, to restore near normal blood PCO_2, and to improve on pulmonary function.

3. Acute Respiratory Failure: Adult Respiratory Failure (ARDS, and Hyaline Membrane Disease of the Newborn (HMD).

The recent collaborative study by NHLBI showed that the membrane lung cannot alter the dismal long term outlook of patients with ARDS receiving optimal pulmonary care by to-day's standards. Furthermore, those fortunate patients to survive a serve bout of ARDS recover fully, with no residual pulmonary disease. We believe those patients now dying of ARDS can benefit by newer concepts in the management of their lung disease, to be discussed below.

In acute respiratory failure of the newborn (HMD) some important innovations have been introduced over the past 8-9 years, reducing the overall mortality to approx. 25%. Those patients who require the use of a mechanical pulmonary ventilator, however, have a protracted hospital course, and show residual lung abnormalities for years.

We believe that mechanical pulmonary ventilation in this select patient population has inadvertently contributed to a progression of their disease to a chronic state, and that other methods of maintaining adequate alveolar ventilation should be considered, i.e. by extracorporeal removal of carbon dioxide.

We have proposed that mechanical pulmonary ventilation can be reduced, or eliminated entirely, if all metabolically produced carbon dioxide is removed by an extracorporeal membrane lung. In this procedure, the subject need not breathe at all. This also illustrates the point that mechnical pulmonary ventilation becomes redundant under those circumstances, and hence pulmonary damage from mechanical ventilation can no longer occur.

Methods Employed and Major Findings:

1. The carbon dioxide membrane lung (CDML) is substantially a spiral coiled membrane lung optimized for carbon dioxide removal. Enhanced carbon dioxide removal can be obtained using either silicone rubber membranes with a long blood path, or using microporous membranes.

Blood compatibility considerations aside, the microporous polypropylene membrane has substantially higher carbon dioxide transport capability than a solid interface membrane, such as a silicone rubber membrane.

We have fabricated a microporous polypropylene membrane lung and

evaluated its performance as a carbon dioxide membrane lung. The carbon dioxide transfer in this lung was in excess of 150 ml/m2/min and the oxygen transport was approx. 100cc/m2/min. This compares with 50 and 70cc/m2/min respectively when using the silicone rubber membrane lung. The main advantage of the polypropylene membrane was a 3 fold rise in carbon dioxide removal. We believe that improved technology will substantially raise carbon dioxide elimination further yet.

Another positive feature of the carbon dioxide membrane lung is its low perfusion pressure. Thus, both parameters can be manipulated to yield results for optimal performance.

2. We have chosen the fetal lamb as the experimental model, long used by neonatologists as the preferred animal model of HMD because of predictable incidence of HMD and because the term lamb is comparable in weight to newborn infants. We have investigated the use of apneic oxygenation as the prime treatment in avoiding the development of HMD in the newborn lamb. In apneic oxygenation, carbon dioxide is removed by the membrane lung, while virtually all oxygen is delivered through the natural lungs without breathing. Some oxygen can also be provided by the membrane lung but at the expense of increaed extracorporeal blood flow; substantially lower blood flows are requried for carbon dioxide removal.

We have been using preterm fetuses (sheep) with a gestational age of 127-130 days (term: 147-150 days). Survival of these fetuses at this gestational age with conventional intensive care is highly unusual. We have performed c-sections, cannulated the external jugular vein, pumped blood through a CDML for carbon dioxide removal, and returned the blood into the umbilical vein while inflating the lungs to a pressure of 15 cm. H_2O and supplying sufficient oygen through a small capillary tube into the treachea to cover oxygen needs.

The animals remained apneic as the blood carbon dioxide level was kept low by the carbon dioxide membrane lung. There was a gradual clearing of both lung fields with sharp cardiac borders by about 6-12 hours of treatment. Their pulmonary compliance gradually improved and rose to near high normal levels. At 24 hours these animals were placed on a mechanical pulmonary ventilator for another 24 hours with further rise in pulmonary compliance, and on room air and with normal bloood gases. The peak inspiratory pressure was 12-15 cm. H_2O.

Lung fluid (fluid at birth inside the lungs and the trachea) was

obtained on delivery, and then after 24 hours on mechanical pulmonary ventilation (i.e. at sacrifice). The minimum surface tension of this fluid was 15-19 dynes/cm, in line with their dated immaturity. There was no significant change in the minimum surface tension from birth, to sacrifice. And yet, these lungs were perfectly normal in terms of pulmonary dynamics, gas exchange, and histology. While presence of a very low surface tension (5-15 dynes/cm) is certaily a sign of biochemical and biophysical maturation, the lack of the same (as measured by an elevated surface tension of 15-19 dynes/cm in this series did not impair the pulmonary ventilation.

More importantly, control animals treated in the conventional manner with mechanical pulmonary ventilatin did develop HMD and died, although their minimum surface tension was in the same range.

Our studies, although incomplete, suggest management of the lungs during the first 24 hours of life as the critical factor that determines whether HMD developes. In the presence of some biochemical and biophysical immaturity in lung development, HMD can be prevented when 24 hours of apenic oxygenation is employed with extracorporeal removal of carbon dioxide. We have consistently avoided HMD in our lamb model. Our findings represent the first successful apprach to avoiding HMD in a preterm lamb of this early gestational age.

These above results, we believe are highly useful in the management of patients with adult respiratory distress syndrome (ARDS). ARDS is a multicause disease, and, behaving as only the lung knows how to, results in severe impariment of alveolar ventilation, and in the clinical syndrome called ARDS. We believe it likely that the relatively simple procedure of extracorporeal carbon dioxide removal by the CDML may allow us to explore other modes of mamagement of ARDS, more in line with the proposed management of HMD.

Importance to Biomedical Research and the Program of the Institute.

1. Progress in extracorporeal circulation and gas exchange requires the adoption of less traumatic blood oxygenators. There has been a trend to wider use of the membrane lung in patient care, very likely due to high quality of membrane lungs now available.

The whole concept of extracorporeal removal of carbon dioxide is a relatively new idea which we feel is a powerful tool in the control of breathing, and in the management of patients with acute respiratory

failure . Over the years, there have been new inroads in improved management of patients with ARDS thgrough the introudction of CPAP, PEEP, IMV, and super PEEP. Interestingly, most of these advances were first used on neonates in the management of HMD, and were only later used in adults. Our ability to avoid HMD in a high risk immature lamb population may quite likely have applicability in both HMD and ARDS.

The use of microproous polypropylene membranesin the CDML has very far reaching implications. It is now possible to design, and to obtain sufficient carbon dixoide removal by a CDML, with a surface area of 1-1/2 - $2m^2$, and at a blood flow of less than 1l/min. There are many important implications. With this efficiency, it becomes technically much simpler to institute, and to maintain low extracorporeal blood flow, for the total or partial control of breathing. Technically, this approach becomes as simple as hemodialysis, if not simpler.

Proposed Course:

1. The CDML will be further optimized for increased performance, improved blood compatibility, and ease of use.

2. We will further explore optimal treatment modes to the prevention of HMD in immature, and premature lambs.

3. We will continue to explore blood compatibility of engineered membranes containing different fillers, and using different processing techniuqes.

Publications:

1. Kolobow, T.: Commentary. World Jounral of Surgery, in press, 1979.

2. White, D. C., Kolobow, T., and Bowman, R. L.: The blood-membrane bioartificial system: Hormone production by human endocrine adenomas perfused with sheep blood. Trans. Amer. Soc. Artif. Int. Organs, in press, 1979.

3. White, D. C., Kolobow, T., Bowman, R. L.: High density tissue culture on microporous membranes perfused by blood. A report of a new bioartificial system. Int. J. Artif. Organs 1: 280, 1978.

4. White, D. C., Trepman, E., Kolobow, T., Shaffer, D. K., Reddick, R. L., and Bowman, R. L.: The microscopic characterization of multinucleated giant cells formed on polymeric surfaces perfused with blood. Artif. Organs 3: 86, 1979.

5. Gattinoni, L., Kolobow, T., Damia, G., Agostoni, A., Pesenti, A.: Extracorporeal carbon dioxide removal. A new form of respiratory assistance. Int. J. Artif. Organs, in press, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01405-05 LTD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (30 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. Techn. Development	LTD NHLBI
	P. Bowen	Biologist	LTD NHLBI
Other:	R. Bonner	Physicist	BEI R

COOPERATING UNITS (if any)
Biomedical Engineering and Instrumentation Branch

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The purpose of this project is the development of a non-invasive method of measuring tissue blood flow by analysis of the spectrum of doppler scattered laser light. The prototype instrument has been greatly improved by a new analysis scheme, a two meter flexible fiber optic probe, and the use of a photodiode detection system. The linearity of the instrument in response to blood flow has been greatly improved and noise has been reduced so the instrument can resolve the instantaneous pulsatile flow of microcirculation. The instrument has been used to monitor blood flow in normal individuals and Clinical Center patients. Preliminary studies of the vascular changes of Sickle Cell Patients and Muscular Dystrophy Patients have been initiated.

S17

Objectives:

To continue development of techniques and applications of a method of continuously measuring the blood flow through small regions of tissue by analysis of the spectrum of coherent light which is doppler-scattered from red blood cells in the tissue microcirculation. The instrument has been shown to be capable of indicating surface blood flow of a great variety of organs, with research applications in vascular physiology and pharmacology, and clinical applications to the study of perpheral vascular disease, burns and grafts, monitoring the tissue perfusion at surgery and under the influence of anesthesia, etc. Specific objectives at this stage are the final development of the instrument and demonstration of applications to a variety of experimental and clinical problems.

Methods:

Instrument Development

The prototype apparatus for monitoring tissue blood flow is continuing to be improved. Through collaboration with Dr. Robert Bonner, BEIB, we have conducted detailed analysis of the doppler spectrum and optimized the instruments linear response to blood flow in a variety of tissues. We have found that a more sensitive linear measure of blood flow can be obtained by calculating the normalized first moment of the Power spectrum or the mean frequency. We have incorporated a fiber optic probe into our system. This probe efficiently transmits the laser light to the subject and the back scattered light to the detection system while providing remotes flexible attachment to the patient. We have also reduced the size and expense of the instrument by using a new photo-diode detection system in place of the photo multiplier.

Major Findings:

1. The new method of calculating flow, the normalized first moment of the power spectrum or mean frequency, appears to be a very sensitive indicator of tissue blood flow.

2. We are able to resolve the wave form of the pulsatile flow in tissue.

3. We are now able to measure flow in a wode variety of tissues. We have normalized our flow parameters to the power of the light back scattered from the tissue and have made the device relatively insensitive to changes in tissue color.

4. The fiber optic probe has greatly improved the flexibility of the instrument and facilitated remote attachment to the patient.

Two clinical protocol exist for studies within the clinical center of NIH. We have made preliminary studies of flow in Sickel Cell patients and have begun a protocol to study Muscular Dystrophy patients during Muscle Biopsy. These studies could lead to conclusions on the relationship of blood flow to abnormal states.

Significance to Biomedical Research and the Program of the Institute:

Laser doppler spectroscopy is a promising method of studying microcirculation of tissues.
It has potential applications not only in the laboratory, but in the clinical study of the peripheral vascular disease, the screening of vasoactive drugs, and the monitoring of patients with unstable circulatory systems.

Proposed Course:

1. Continue development of the instrument, in collaboration with BEIB or industry.

2. Conduct clinical trials to establish the instrument as a useful clinical and diagnostic tool.

Publications:

1. Stern, M.D., Lappe, D. Bowen, P.D., Chimisky, J.E., Holloway, A., Jr., Keiser, H.R., and Bowman, R.L.: Continuous measurement of tissue blood flow by laser doppler spectroscopy. Am. J. Phys. 232: (4) H441-H448, 1977.

2. Stern, M.D., Bowen, P.D., Bowman, R.L., Stein, J.H.: Measurement of renal cortical and medulary blood flow by laser doppler spectroscopy in the rat. Am. J. Physiol. 236: F80-7, 1979.

3. Bonner, R.F., Bowen, P.D., et al.: Real time monitoring of tissue blood flow by Laser Doppler velocimetry. Proceedings of the Electro Optics Symposium. 1979.

4. Bonner, R.F., Bowen, P.D., et al.: A Non-invasive Laser Doppler and radiometric monitor of cutaneous and skeletal muscle microcirculation. Neurosciences: Abst. 1979 Meetings.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01407-16 LTD

PERIOD COVERED
October 1, 1978 to September 30, 1979

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:	Raymond F. Chen	Senior Investigator	LTD NHLBI
	Robert Meeks	Postdoctoral Fellow	LCB, NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, LTD, Bethesda, Maryland 20205

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)

Fluorescence methods were used to study certain problems of concern in biomedical sciences. The areas investigated included: 1. The effect of Vitamin A analogs on the fluidity of cell membrane lipids in relationship to the toxicity of the analogs. 2. The binding of metal ions to tryptophan peptides, as determined by fluorescence quenching. 3. The functional integrity of phospholipid bilayer membranes, as affected by heating or treatment with phospholipases. The changes in membrane structure were followed by a fluorescent dye leakage method. 4. The effects of salt and phospholipids on the activity of a number of enzymes such as alcohol dehydrogenases and glutamate dehydrogenase.

520

PHS-6040
(Rev. 10-76)

Objectives:

The purpose of the project is to apply fluorescence methods to interesting problems in biochemistry to elucidate both the problem and to advance the fluorescence techniques employed. In doing this, the laboratory extends the methodological expertise developed here in the past and demonstrated the range of problems which are amenable to such methods.

Methods Employed:

Fluorescence spectra, quantum yields, and polarization were measured on Ami-Aminco-Bowman spectrofluorometers. Fluorescence decay kinetics were followed with a TRW Instruments nanosecond decay apparatus or with the ORTEC 9200 photon counting spectrometer. Computer calculations were performed with the MLAB program on the PDP-10 computer. The chemical specimens were supplied commercially or by collaborating scientists.

Major Findings:

1. As part of a program to investigate the correlation between toxicity of Vitamin A derivatives and their effects on membrane integrity, we have performed a study using fluorescence polarization of a membrane probe, 1,6-diphenylhexatriene (DPH). In this technique, rat erythrocyte membranes were incubated with DPH which preferentially binds to the bilayer leaflet. The molecular rotational mobility of the DPH was followed by measuring its fluorescence polarization and lifetime. Detergents, which are known to disrupt the membrane were added and the DPH polarization was found to decrease in a dose dependent manner. The effect of detergents was mimicked by certain Vitamin A analogs. It was found that the concentration of analog needed to disrupt the membrane was inversely correlated with the toxicity of the compound. Retinoic acid was a strong membrane-active compound, more so than a number of other active Vitamin A analogs such as retinal, but weaker than some synthetic retinoids. The results suggest that it will be possible to predict the toxicity of a retinoid from its effect on membrane microviscosity in vitro. These results are important in assessing compounds now being synthesized for cancer chemoprevention, a promising area in retinoid pharmacology.

2. The integrity of phospholipid liposomes was assessed by a new fluorescent dye technique developed initially by W. Hagins in collaboration with J. Weinstein et al in the N.C.I. These people found that a dye, 6-carboxyfluorescence (6CF), at a concentration of 0.2 M was virtually nonfluorescent and could be incorporated into small liposomes (300 A diameter) by sonication in the presence of phospholipids. When these dye-containing liposomes are then lysed, the fluorescence increases markedly thereby providing a sensitive optical probe of membrane integrity. We have investigated the 6CF-liposome system to see why concentration quenching occurs, and have applied the system to the study of melting temperatures of various phospholipid membranes; i.e.,

the temperature at which the liquid crystalline →gel transformation occurs. From quantum yield, lifetime and absorption spectral measurements it was concluded that the quenching occurs because of formation of non-fluorescent dye aggregates, probably predominantly the dimer, and because of energy transfer to nonfluorescent dimers. A number of "melting curves" could be determined for various phospholipid combinations using the fluorescence intensity of the 6CF-liposomes, which increased markedly at the phase transition temperature. The usefulness of the 6CF-liposomes system in studying phospholipase mechanism was demonstrated by following the rate of lysis of the liposomes by the enzymes as a function of activators such as serum albumin and divalent metal ions. It was shown that some liposomes treated with phospholipase A_2 of snake venom do not lyse until serum albumin is added, presumably to remove the hydrolysis products, fatty acid and lysolecithin. Phospholipase A_2 activity declined sharply with time, presumably due to inhibition by fatty acids, which are products of the reaction. Serum albumin relieved the inhibition, but was itself an inhibitor of other phospholipases, namely, phospholipase A_2 of pancreas and phospholipase C of bacilli. These findings demonstrate the usefulness of the dye system in studying the differences in mechanisms of action of different phospholipases.

3. Phospholipid liposomes were found to activate alcohol dehydrogenases from yeast and horse liver. In contrast, these liposomes inhibited the activity of liver glutamate dehydrogenase. The mechanism and possible significance of such modulation of activity were investigated. In other instances of enzyme activation, the mechanism seems to be an increase in the binding constant for the substrate; i.e., a decrease in the K_m or Michaelis constant. Our data seem to show that the K_m is little altered, and V_{max} is increased. Phospholipids also seemed to affect enzyme stability, suggesting that the mechanism of activation involves a physical conformational change in the enzymes. The rotational mobility of the enzymes in the presence and absence of phospholipids was assessed by the fluorescence polarizational technique in which the proteins are labeled with a suitable dye. The significance of these findings is that phospholipids may constitute a normal control mechanism for modulation of enzyme activity, since even "soluble" enzymes are often in contact with membranes. Several other enzymes were examined after working out their respective assays, to see if they also were either activated or inhibited by phospholipids, but so far the above-mentioned enzymes are the only ones showing this effect.

4. We have continued working on the determination of binding constants by fluorescence quenching, using the system Cu^{++} + tryptophanyl peptides. The most common method of determining metal-ligand binding constants is potentiometric titration in which the displacement of hydrogen ion by metal ion is used to calculate the association constant:

$$Me^{++} + LH^{+} \rightleftharpoons MeL^{++} + H^{+}$$

where Me^{++} and L are metal ion and ligand, respectively. The fluorescence quenching method makes use of the fact that tryptophanyl ligands are fluore-

scent, while the metal complex is nonfluorescent if the metal ion is one such as Cu^{++}. For binding of Cu^{++} to tryptophan itself, we have obtained an association constant of 3.9×10^{8} in agreement with literature values. However, for peptides containing tryptophan, there is no reason to expect that the association constants obtained from fluorescence quenching will agree with those in the literature obtained by potentiometric titration. This is so because binding may occur through peptide and ionized carboxyl groups with no alteration in hydrogen ion concentration. Work under way involves determining the binding constants for various trytophan peptides with Cu^{++} and Ni^{++}, and some data have been obtained showing feasibility of extending this method to peptides of tyrosine and phenylalanine.

Significance to Biomedical Research and the Program of the Institute:

Fluorescence methods have been used to promote an understanding of the mechanism of toxicity of Vitamin A compounds, membrane structure and function, enzyme mechanism, and the interaction of metals with biological compounds. These studies are of significance to the understanding of physiological processes and promote the institute's policy of developing methodology for use in biomedical research.

Proposed Course:

The work on retinoids has been completed and a poster was presented at the meeting of the American Society of Biological Chemists. The detailed paper is in the final stages of production. A paper on the dye-liposome data, including phospholipase interaction, is also being finalized. The data on metal ion binding and on enzyme interaction with phospholipid should be ready for publication in the next few months. We intend to continue to work on these problems and to extend our observations on liposome structural changes as determined by fluorescence.

Publications:

Robert G. Meeks and Raymond F. Chen, "The Effect of Membrane Detergents and Retinoic Acid on Membrane Microviscosity", Fed. Proc. 38: 540 (Abstract # 1645), 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01408-14 LTD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Methodology in Fluorescence Measurements

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

PI: Raymond F. Chen — LTD NHLBI

Other: O. Alabaster — LP C

COOPERATING UNITS (if any)

Laboratory of Pathology, National Cancer Institute

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	.5	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

1.) The properties of fluorescent derivatives of ~~o-phthaldiadehyde~~ were studied in order to facilitate the use of this compound in the assay of amino acids and the labeling of proteins. The reaction rates and fluorescence parameters were measured and discussed in relation to other reagents and methods for amino acids.
2.) Various solvents commonly used in phosphorimetry were tested for inhibitory effects on alcohol dehydrogenase. Some alcohols were inhibitory, while some salts activated the enzyme, so that the interpretation of phosphorescence data should include the possibility of conformational change due to the matrix.
3.) An artifact has been found and characterized in the operation of the Los Alamos Flow Cytometer and cell sorter. When cell volume is being measured in this instrument, the fluorescence of the suprovitally stained cells decreases due to electrolysis. The mechanism of bleaching apparently involves generation of chlorine at the anode.

834

Objectives:

The purpose of this paper is to develop fluorescence methods for assaying biological substances and to evaluate the problems and complications in various techniques involving luminescence. In this way we extend the usefulness of luminescence techniques as applied to biomedical research.

Methods:

The instrumentation used to measure fluorescence parameters are basically those which have been developed in this laboratory over the years. The Los Alamos Flow Cytometer which was studied was that belonging to the Laboratory of Pathology, National Cancer Institute.

Major Findings:

1. One study was concerned with the mechanism of the o-phthaldialdehyde (OPA) reaction for primary amines. This work was continued from the previous year, completed, a paper was written and published. In summary, the fluorescence properties of the products formed by reaction of OPA with amino acids and their derivatives in the presence of thiol compounds have been measured. Since the emission spectra, quantum yields, and lifetimes depend on the primary amine and thiol compounds used, the product which fluoresces must incorporate parts of three molecules, including the OPA. The fluorescence quantum yields of OPA derivatives of the amino acids ranged from 0.33 to 0.47 if 2-mercaptoethanol is the thiol compound. The fluorescence lifetimes were 18-20 nanoseconds. OPA derivatives of amides and peptides had much lower quantum yields due to quenching by the carboxamide group. This quenching was relieved by detergents and Dimethylsulfoxide. Lysine, which has 2 amino groups, reacted with OPA to give either a monosubstituted derivative with a normal fluorescence, or a disubstituted derivative which was largely quenched. Stopped-flow fluorescence measurements could resolve two separate rate constants for reaction of the two amino groups. The stability of the OPA derivatives was also investigated.

2. An artifact in the operation of the Los Alamos Flow Cytometer and Cell Sorter was investigated. The artifact was important because it alters the results of flow cytometry, a technique which is widely used in laboratories throughout the world. It was noticed that the instrument gave spurious results on cell distributions whenever the current was turned on to measure the Coulter electronic cell volume. Using a cuvet fitted with two platinum electrodes by Dr. G. Vurek, we observed electrolysis of various solutions whenever a current comparable to that used in the instrument was turned on. The cells usually are stained with mithramycin, ethidium bromide, or propidium iodide, but the fluorescence of these dyes rapidly fades under the conditions of electrolysis. Dye solutions were passed through the instrument in the presence and absence of Coulter current, and the result showed dimming of the fluorescence in the absence of cells as well as in their presence. The chan-

ges in spectra were mimicked in the large cuvette by passing a current through the solution (4 ma for 5 to 15 min.). Dry chlorine gas also rapidly bleached the dyes. Running the stream through the flow cytometer at higher speeds reduced the effect of Coulter current. These results show that probable electrolysis is the cause of dye bleaching during flow cytometry, and by publishing these results it is hoped that instrumental design will be influenced.

3. In measuring the phosphorescence of proteins, solutions usually are made up which will be clear at 77°K. In order to obtain such clear glasses, ethylene glycol and glycerol are usually added to aqueous solutions and it is assumed that no conformational changes in the proteins occur. We have tested this hypothesis with liver alcohol dehydrogenase, since it has been reported in the literature that glycerol and other polyhydric alcohols cause conformational changes in glutamate dehydrogenase. We found that alcohol dehydrogenase can utilize several of the commonly used alcohols as substrates, including glycerol, ethylene glycol, and propylene glycol. However, the presence of ethylene glycol, which is a poor substrate, grossly inhibits the oxidation of ethanol and the inhibition is only partly competitive with ethanol. One concludes therefore that ethylene glycol interacts with the enzyme both at the active site and elsewhere to produce inhibition. The polyhydric alcohols also seem to unfold part of the enzymes as judged by progressive exposure of the tryptophans as monitored by changes in their intrinsic fluorescence. We conclude, therefore, that the organic solvent matrices often used for phosphorimetry of proteins are at least mildly denaturing and one should use caution in interpretation of results obtained in such matrices. In contrast, aqueous snows are sometimes used for protein phosphorimetry. Such snows are made by freezing aqueous solutions containing 0.1 - 0.2 M NaCl or KCl. Such solutions at room temperature are not inhibitory to enzyme activity, and it is more likely that such matrices provide phosphorescence information which is more representative of the native state of the protein than do the organic solvent matrices.

Significance to Biomedical Research and the Program of the Institute:

Since the first spectrofluorometer was developed in this institute some 20 years ago, the laboratory has continued to extend and develop methods and techniques used in luminescence spectrometry. By continuing this work we have helped fluorescence and phosphorescence methods become very useful in biomedical research.

Proposed Course:

While one aspect of the OPA problem is complete, we have much data on the rates of reaction of OPA with various amino acids, and hope to continue work on this area until a coherent story can be made for publication. We also plan to complete some work on the interaction of a membrane probe with proteins. This work so far has demonstrated that there are some complications in the use of membrane probes; namely, that such probes interact not only with membranes and lipids, but also with proteins. The work with phosphorescence matrices should

also be completed and written up for publication.

Publications:

1. Chen, R.F., Scott, C., and Trepman, E.: Fluorescence Properties of o-Phthaldialdehyde Derivatives of Amino Acids, Biochim. Biophys. Acta, 576 (1979) 440-455.

2. Alabaster, O., Hamilton, V.T., Bentley, S.A., Glaubiger, D., Shackney, S.E., Skramstad, and Chen, R.F.: "Flow Cytometric Measurement of Electronic Cell Volume Can Chemically Degrade DNA Fluorochromes," Abstract presented at Cell Kinetic Society Meeting, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01411-13 LTD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Blood Flow Measurement Using Nuclear Magnetic Resonance Techniques

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

PI:	V. Kudravcev	Engineer	LTD NHLBI
	R.L. Bowman	Chief, LTD	LTD NHLBI
Other:	A. Sances	Professor and Chairman Biomedical Engineering	Medical College Wis.
	J.H. Battocletti	Associate Professor Dept. of Neurosurgery	Medical College Wis.

COOPERATING UNITS (if any)
Medical College of Wisconsin, Milwaukee, Wisconsin

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The principles of nuclear magnetism have been applied to the measurement of blood flow utilizing the proton of the water fraction of blood. Specifically, the nuclear magnetic resonance phenomenon (NMR) is used to enhance the detection of these protons once they have been polarized by a strong, steady, magnetic field.

Two different systems have been used: 1) A two magnet system using a high-level field polarizer and a low-field homogeneous detector, 2) A one magnet system using a high-level, homogeneous field which serves as polarizer and detector. Both systems can employ an erasing field at a specified point between polarizer and detector to determine the proportion of blood passing through the erasing field.

A specific aim is to provide the necessary instrumentation for measuring regional intracranial blood flow to facilitate studies of intracranial atherosclerosis and stroke.

PHS-6040
(Rev. 10-76)

PROJECT DESCRIPTION:

The project consists of (1) LTD electronics section devoted to the development and refinement of special circuits and modes of operation; (2) a contract facility cooperating in, and contributing to the circuit development and concerned with the testing and evaluation of the experimental apparatus on test models, animals, and human volunteers in preparation for clinical application.

Last year, LTD proposed a method of weak NMR induction enhancement by double resonance-proton plus electron resonance. This new Kudravcev method is based upon the utilization of a well-known phenomenon of dynamic polarization, the "Overhauser effect." The relaxation of the electron resonance is achieved by fluctuating the magnetic field in the proton resonance region by the inflow of disoriented protons from upstream. The field of disoriented protons from the inside of the flow probe penetrates into the outside electron resonance probe. Relaxation in the electron resonance source is produced (if a proper frequency of fluctuating field is utilized) and causes a strong change of the detector output. The enhancement of the resonance detector output may improve the sensitivity of the blood flow measurement devices. At the present time, a relatively low sensitivity of these devices is the main factor limiting wider application in the clinical practice.

A "hybrid" exciter-receiver (nuvistors and solid-state elements) was constructed for the ESR/NMR experiment. It is in process of final testing and adjustments. An experimental NMR/ESR probe was constructed using DPPH as the ESR material. On the basis of testing this probe, it was determined that the helical resonator probe would be more efficient. This probe will be given to the MCW laboratory which has the necessary facilities.

Our solid-state superheterodyne receiver was modified in such a way as to cover frequencies used in double resonance experiments. Because of the commercial I.F. amplifier used in this receiver was found to be very noisy and overload-prone, a new I.F. amplifier design will be produced in collaboration with the Biophysics Lab at MCW.

Other work performed at LTD included the following:

(a) Testing and utilization of the 2.8 MHz single-sideband receiver constructed by MCW.

(b) Various electronic refinements of our NMR blood flow research apparatus to achieve better sensitivity and stability.

At the medical college of Wisconsin, the main thrust of work has been directed toward cooling down and operating the superconducting magnet, and perform-

ing a number of NMR experiments at both 21.4 MHz (5026 gauss) and 75 MHz (17,615 gauss) in vitro and in vivo.

The superconducting magnet and its control console perform satisfactorily. The homogeneity is within the limits specified by the manufacturer. After repairing a leak in the vacuum chamber of the magnet, the normal helium consumption is one-half of the specified amount. However, transfer and other losses of liquid helium account for 58% of the total liquid helium consumed. It would probably be cost effective to purchase a closed-cycle helium liquifier not only to save this 58% money loss, but to conserve helium which is a limited resource.

Transmitters, receivers, precision rectifier detectors, flat crossed-coil NMR probes, and a modulation coil were designed and built for the two frequencies of operation. An aluminum slideable track was constructed to allow the subject to be inserted into the 63 cm I.D. bore of the magnet. These were successfully tested in the 39-day run of the superconducting magnet. The following summarizes the various tests performed and the results obtained in the detection of pulsatile (arterial) flow:

a. In vitro tests demonstrate that NMR output is proportional to volume flow rate up to a threshold level. Furthermore, pulsatile flow in a tube spaced 24 mm from the flat crossed-coil probe can readily be detected.

b. Both systems readily detect flow in the posterior tibial artery in "real time". The dichrotic notch and negative flow can be seen on the NMR output signal. The 75 MHz signals have a higher signal-to-noise ratio than the 21.4 MHz signals, as expected.

c. Carotid signals from the chimpanze are readily detected using the 75 MHz system in both "real time" and "signal averaged."

d. NMR signals from the carotid, temporal, and other arteries in the human head are readily detected at 21.4 MHz. Good "real time" signals are obtained from the carotid; however "signal averaging" is required for good detection of flow in other arteries in the head.

e. Very large "real time" flow signals are obtained from the region of the ascending aorta at 21.4 MHz. A "Valsalva maneuver" indicates that the signal is a true flow signal and not motion artifact.

f. Twelve rhesus monkeys were exposed to a 20,000 gauss steady magnetic field for a period of 68 hours. Complete blood tests and somatosensory evoked potentials were taken on each. A preliminary study of the data indicates that there were no ill-effects. A more exhaustive statistical analysis indicates that the only significant change was in the type of white blood cell present after exposure. However, the same changes were observed in four monkeys which underwent the same routine as the twelve, but with no

magnetic field. Therefore the morphologic change of white blood cells must be attributed to stress.

g. A two-magnet setup was tested, using a 100 kHz system as the detector and the 20,000 gauss superconducting magnet as the pre-magnetizer. Whereas very strong NMR signals were obtained with water flowing, no discernible signal could be obtained with blood flowing. This confirms the low value of T_1 for blood in weak fields (e.g., between the pre-magnetizer and the detector).

OBJECTIVE:

To develop noninvasive methods of measuring and tracing regional cerebral blood flow (rCBF) using nuclear magnetic resonance techniques.

METHODS EMPLOYED:

The hydrogen nucleus of the water fraction of blood has a magnetic moment which can be acted upon and sensed by external steady and alternating magnetic fields.

Magnet, coils, electronics and display equipment are combined to provide the necessary fields and detection circuitry as described in previous reports.

PROPOSED COURSE:

1. LTD will continue the development of the ESR/NMR system for the enhancement of signal-to-noise ratio. MCW will construct a helical resonator probe and I-F amplifier for this work.

2. Complete the evaluation of the 2.8 MHz single sideband receiver at LTD.

3. MCW will make certain modifications of the 21.4 MHz and 75 MHz systems in consultation with LTD. These include:

   a. Design and contruction of a two-phase synchronous detector module to improve the detection efficiency.

   b. Design and construct a new modulation coil to lessen eddy-current losses and to increase the modulation field.

   c. Design and construct new and larger flat crossed-coil probes to allow deeper vessel in the brain to be measured.

4. In the next run of the superconducting magnet, emphasis will be placed on the development of a calibration technique for the flat crossed-coil probe.

5. Work will be started to develop the active tag method of measurement of steady, or venous, blood flow, in both the 21.4 MHz and 75 MHz systems.

6. It is hoped that confidence will be gained from the rhesus monkey exposure tests to allow us to perform brain flow measurements at 75 MHz sys- (17,615 gauss) without the apprehension we had during out last run of the superconducting magnet. Although we did not expect any deleterious results on the 12 monkeys which were exposed for 68 hours, and since there was no evidence in the literature of deleterious effects of steady magnetic fields up to 20,000 gauss, we were still concerned about undue exposure to subjects.:

Thus, with this confidence we hope to make a large number of flow tests at 75 MHz on human volunteers and hospital patients during the coming year.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

A blood flow measuring system that could follow changes in regional cerebral blood flow has been identified as a major problem in cardiovascular research for pathology leading to stroke. The noninvasive nature of the NMR blood flow measurement has already been applied linically to the measurement of limb blood flow. These techniques are now being extended for rCBF since they can be used for chronic observation or frequent use without morbidity. Ultrasonic methods useful elsewhere in the body are rendered loss promising by the high attenuation of ultrasonics by the skull.

PUBLICATIONS:

1. Battocletti, J.H., Halbach, R.E., Sances, A., Jr., Cusick, J.F., Salles-Cunha, S.X., Bowman, R.L. and Kudravcev, V.: Cerebral blood flow measurement: NMR flat crossed-coil probe. Proc. 31st Annual Conference on Engineering in Medicine and Biology, Atlanta, Georgia, 20: 79, 1978.

2. Battocletti, J.H. Halbach, R.E., Sances, A., Jr., Cusick, J.F., Salles-Cunha, S.X., Bowman, R.L. and Kudravcev, V.: Cerebral blood flow measurements: NMR flat crossed-coil probe. Ibid, 20-79, 1978.

3. Battocletti, J.H., Bowman, R.L., Halbach, R.E., Salles-Cunha, S.X., Sances, A., Jr., Evans, S.M., Bernhard, V.M. and Herbert. L.A.: Peripheral blood flow measurement using a nuclear magnet resonance flowmeter. J. Clinical Research 26(5): 695A, October, 1978.

4. Battocletti, J.H., Halbach, R.E., Sances, A., Jr., Larson, S.J., Bowman, R.L. and Kudravcev, V.: Flat crossed-coil detector for blood flow measurement using NMR, Med & Biol Eng & Comput, 17: 183-191, March 1979.

5. Halbach,R.E., Battocletti, J.H., Sances, A.,Jr., Bowman, R.L., Kudravcev, V.: Cylindrical crossed-coil NMR limb blood flowmeter, Rev. Sci Instrum 50(4): 428-434, April 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01413-17 LTD

PERIOD COVERED
October 1, 1978 to September 30, 1979

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

P.I. :	R. L. Berger	Chief, Biophysical Instrumentation	LTD:NHLBI
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	G. Liesegang	Staff Fellow	LTD:NHLBI
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	p. Bungay		BEIB:DRS
Others:	J. Forehlich		Nat. Ageing Inst.
	J. Pochobradsky		
	W. Friauf		BEIB:DRS
	C. Gibson		
	H. Hopkins		Georgia State U.
	N. Davids	Consulting Mathematician	Penn State U.

COOPERATING UNITS (if any)

Biomedical Engineering and Instrumentation Branch, DRS, NIH
Thermometry Section, National Bureau of Standards

LAB/BRANCH
Laboratory of Technical Development

SECTION
Section on Biophysical Instrumentation

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The fast thermal stopped-flow apparatus has been rebuilt to eliminate the temperature rise produced upon stopping. By coupling the drive syringes to two identical output syringes a zero pressure gradient across the observation tube is produced upon stopping. The total temperature artifact due to flow and stopping is now less than 1 millidegree C. Utilizing FEST (Finite Element Simulation Technique) sensor response of 7 milliseconds to 1/e has been corrected to 3.5 milliseconds and is limited mainly by the 3 to 4 millisecond dead time of the instrument.

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Project Description

Objectives:

The objectives of this project are to develop new instrumentation methods, data handling techniques and theoretical treatments for the physiochemical study of the thermodynamics,kinetics and thus the mechanisms of enzyme action in solution and in the intact cell or cell membrane. In particular, to develop methods and instruments to study, in collaboration with other laboratories, the reactions of hemoglobin with the respiratory gases both in normal state and as modified by the changes of physical factors, small molecules, various metabolites, and genetically, such as in sickle cell anemia. The ractions 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 enzyme action are those of pre-steady state chemical kinetics and thermodynamics. Measurements of the appropriate parameters are made by developing the necessary equipment to mix solutions rapidly and follow the course of the resulting chemical reaction by optical, thermal, glass electrode, etc., detectors. In general, equipment is not available, either in the literature or commercially, for investigations in this area. Such apparatus is conceived and designed in this laboratory, together with consultants, construction being caried out wherever most appropriate; i.e., in our shops or by commercial firms, special university facilities, or at several special research laboratories such as the Jet Populsion Laboratory. In pursuing these investigations, a wide variety of physical parameters must be studied, which leads to the need for an understanding of the underlying physical theory governing the reactions. Expert consultants and collaborators are brought in to assist in the design, analysis, and evaluation of the equipment,. particularly as it is appleid to certain specific enzyme systems under investigation.

Major Findings:

The major development this year in instrumentation for the study of fast enzyme kinetics has been the completion of th fast thermal stopped-flow apparatus. This instrument has a time resolution of 3 milliseconds, a sensitivity of 10^{-4}°C, i.e. for a one kilocalorie/

Mole heat of activation this represents a sensitivity of 10^{-4} moles/ml. Since most enzymes have a reaction rate such that in one millisecond half of the reagent is used up at an enzyme concentration of 10^{-6} moles per ml and an activation heat between 2 and 12 kilocalories per mole. We are roughly 10 to 50 times less sensitive than we would like to be. In order to overcome this difficulty we have been carrying on a joint development program with the Thermometry Section of NBS. These sensors have been primarily the capacitance type but thin film resistance units have also been explored. At present, capacitance thermometers have been produced which have at least a 10 fold increase in both sensitivity and speed response. However, the application of a protective coating thin enough to not materially effect the speed of response yet pin hole free so that the unit can be used in 0.1 M salt solutions has not been successful. Further work on this problem is in progress. To test the present system on an important reaction whose kinetics are not known, we have used the reaction of Carbon Dioxide with Glycl-Glycine as a known reaction and the reaction with hemoglobin as the unknown. Preliminary results indicated general agreement with the Roughton-Rossi data using bovine hemoglobin (where 100 ml of 5 mM hemoglobin had to be used versus 0.25 ml for this apparatus). The reaction of 2.3 DPG with hemoglobin is also under study. Both reactions were somewhat obscured by a pressure artifact in the thermal flow system which arises due to the adiabatic compressibility of water. This artifact has now been eliminated by the construction of a new flow system wherein the driving syringes are coupled to two identical stopping syringes thus producing a zero-gradient across the observation tube at stopping. By utilizing a finite element response correction program we are able to correct a 7 millisecond time response thermistor to at least 3 milliseconds as can be seen from the following table of the results of carbon dioxide-glycl-glycine reaction compared to continuous flow results.

TABLE 1

	CO_2 + Gly-Glyc		Our Measurements	
Con CO_2 M	Con G-G M	T1/2	K(L/M-S)	K(Continuous Flow)
10	50	14.0	2,101±40	2030±16
10	100	7.0	2,070±40	2030±16
10	200	3.5	1,970±40	2030±16

In order to suggest improvements in the design and operation of an instrument, one needs to obtain information about the various pehnomena which contribute to the signal detected by the sensor. In our case we have heat generated by viscous dissipation during flow, the chemical reaction, temperature gradients in the flow system, temperature effects due to the adiabatic compressibility of water, and thermal conduction. All of these effects have been incorporated into a computer program using the finite element simulation technique (FEST) which simulates the action of the calorimeter from the start of flow to stopping and until the reaction has been sufficiently completd for end-point determination to at least .1%. Since this is a simulation in time the acceleration of the fluid and the temporal behavior of the mixing is also included in the calculation. By performing these calculations we have been able to obtain a better understanding of phenomena contributing to the thermistor bridge output.

The available instrumentation developed in this laboratory, for the study of enzyme reactions, now consists of a variety of stopped-flow instruments whose time resolution ranges from 200 microseconds to 1.5 milliseconds with optical, pH, and thermal detectors available as sensors of the reactions under study. These instruments, by necessity, generate large amounts of data. In addition to the data acquisition, smoothing, and sensor corrections that are needed befor the data can be fitted to a theoretical model, there are both instrument control functions as well as extnsive fill handling capabilities needed. Lastly, a theoretical modeling and automatic curve fitting procedure was needed for determining rate constants, etc. The use of microprocessors has been extensively exploited by this laboratory in conjunction with the instrument development program. A unique laboratory computer system has been developed, called DLAB, which can run on any computer but does in fact run on the NIH PDP-10, our own MDS-800 8080 computer and our S100 bus structure 8080 Sol III computer. These later two microprocessor systems utilize 64 Kbytes of 8 bit core and dual floppy disks. Programs are written in Fortran and assembly language. The present structure of the system and its data acquisition, control, modeling and curve-fitting capabilities are shown in Figure 1. User generated functions can also be used with th curve-fit (taken from MLAB). Application of this system to the hemoglobin modeling data has demonstrated the extreme need for very accurate data during the first 15% of the reaction. It is in this region that discrimination between models seems to be most important. As an adjunct to the stopped-flow data on the reaction of oxygen with hemoglobin, work has continued on an attempt to develop a flash photolysis system with a one microsecond time resolution. This unit is of the dual wavelength type and operates in the near-infra red where the absorption coefficients of hemoglobin are two orders of magnitude lower than in the visible, thus permitting us

to work with very high concentrations, similar to what exists in the red cell and with red cells themselves. However, the high absorption coefficient of methemoglobin puts a stringent requirement on the preparation of Hb since the MET must be kept below 1% if the correct changes of oxyhemoglobin are to be followed.

In many biological applications, for instance muscle studies, chelating agents are used to control the concentration of metal ions, such as Ca. Ethylene glycol bis(amino ethyl either) tetra-acetic acid (EGTA) is used for situations in which a preferential chelation of calcium is required in the presence of magnesium ions. The log of the stability constant is 11.0 for calcium and 5.2 for magnesium; these values can be compared to the corresponding values for metal ion complexation by EDTA, which are 10.7 and 8.7 for Ca and Mg respectively. In muscle studies where the calcium ion concentration varies as a function of time, the rate of calcium uptake by EGTA is the important parameter to know in order for EGTA to act as a viable calcium buffer. Kinetic studies of calcium ion complexation by EGTA were investigated using stopped-flow and temperature-jump techniques. Investigations were performed within the pH interval 4.5 to 7.5, using appropriate hydrogen ion indicators to monitor the reaction. The kinetic data was fitted to the mechanistic scheme.

$$EGTAH_2^{2-} + CA^{2+} \xrightarrow{ko} (CAEGTA)^{2-} + 2H^2$$

where ko may comprise several reaction steps. This average rate constant for complexation, k_o, was found to vary with hydrogen ion concentration above pH 5.5. From this pH dependency (above pH 5.5) it is hypothesized that both the mono- and di- protonated forms of EGTA are complexing Ca^{2+}. At neutral pH the value for k_o is $3.0 \times 10^6 M^{-1}S^{-1}$ (25.0°C) well below the diffusion controlled limit. This slow rate is thought to be due to the braking of an intramolecular H-bond in EGTA prior to metal-ion complexation. Given a stability constant of 5.0×10^6 (pH 7.0) for the EGTA-Ca complex, a value of 0.5 sec^{-1} is obtained for the dissociation rate constant of the complex. Work at 8°C indicates the rate drops by a factor of 5.

To allow flash photolysis or photoactivation experiments at various wavelengths, the output of the laser equipment was made turnable. Between the maximum reflector and the dye cavity were placed a pair of 60° prisms orientated a minimum deviation. This produces a dispersive element within the laser cavity and thus allows turning of the laser output wavelength; arrowing of the laser output to a few nonometers is also achieved. A range of dyes is avilable to cover the principle regions of interest within the visible spectrum. To further extend the versatility of the equipment, two second harmonic generation crystals have been obtained to be used in conjunction with the tuned output.

The first crystal convets 560 nm light to 280 nm light which overlaps the tryptophan/tyrosine absorption of proteins, in particular, studies are being carried out on the temperature dependence of phosphorescence produced in various enzymes, for example, liver alcohol dehydrogenase. Phosphorescence has been detected at low temperature and the observation cell is being modified to permit the temperature dependence to be studied. A second crystal produces 340 nm light pulses which are to be used to study the feasibility of producing an instrument to detect submicro amounts of cholesterol. After enzymatic oxidation of cholesterol, hydrogen peroxide is produced, which upon reaction with p-hydroxypenyl acetic acid a fluorescent product is formed. It is this fluorescent product which is to be excited by the 340 nm light.

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, and computer coupled control, data acquisition, correction and model testing on line. Work will continue on a new multiple mixing, continuous flow system to be usd with acid and low temperature quenching and filtration. Completion of the laser flash duel wavelength system will be carried out. Thermal sensor development with NBS will be stepped up with particular attention paid to coatrings.

Publications:

1. Berger, R. L., Balko, B., Bowen, P., Paul, R., and Hopkins, H. P., Jr.: Frontiers of Biological Energetics, Vol. 1, Ed. P. L. Dutton, A. Scarpa, Academic Press, 1978, pp 698-706.

2. Pochobradsky, J., Balko, B., and Berger, R. L.: The Effect of the Pressure Pulse on Solution Temperature in Stopped-Flow Calorimetry, Anal. Letters, Vol. A1162, 1097-1107, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01414-07 LTD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Development of Microcalorimeters and Differential Apparatus for Biochemical Reaction Sutdies

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

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	H. Hopkins	Professor of Chemistry	Georgia State Univ.
	C. Mudd	Mechanical Eng.	BEIB:DRS
	L. Thiebault	Mechanical Eng.	BEIB:DRS
	W. Friauf	Chief, Elec. Eng. Sect.	BEIB:DRS
	T. Clem	Chief, Elec. Eng. Sect.	BEIB:DRS
	M. Sappoff	Chief & Pres. Elec. Eng.	Thermometrics

COOPERATING UNITS (if any)

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Thermomentrics, Inc., Edison, N.J.

LAB/BRANCH
Laboratory of Technical Development

SECTION
Section on Biophysical Instrumentation

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The differential batch calorimeter has been modified to eliminate rotational thermal artifacts produced at mixing. FEST indicates chemical reactions with rates slower than 0.2 sec $^{-1}$ can be determined with the present analysis. A new thermistor linearizer using a microprocessor has been built and tested with an NBS standardized thermistor accurate to + 0.01°C. The new instrument hs demonstrated the feasibility of this approach but due to front end problems reproducibility at the + 0.005°C level can only be achieved at 50 microwatts which is too high for cuvette use since the dissipation of the thermistor is 300 microwatts per degree.

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PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

Virtually all chemical reactions produce heat and calorimetry has long been used to investigate them. For biological use, however high sensitivity, small volumes of reactants, and short equilibrium times are needed. It is the objective of this project to develop such an instrument for use in the time range of a few seconds to 1 or 2 hours.

Methods Employed:

Initial designs are constructed in this laboratory with special assistance from commercial firms in the construction of sensors; contracts are let, where warranted, for the development of completed instruments with refinements that would tax our own facilities. The instrument is then tested in conjunction with other interested biochemical calorimetrists utilizing appropriate enzymatic and cellular reactions.

Major Findings:

During the past year the differential batch microcalorimeter has been improved to the point that the long term stability is 0.2 microwatts. Rotation artifacts have been virtually eliminated. A detailed testing of the finite element simulation and data reconstruction program is presently being carried out to determine how fast a reaction can be studied. The 1/e response of the calorimeter is 40 seconds. Electrical pulses, in the form of step functions, have so far been determined to 4 seconds. This means that at the moment the rate constant of a chemical reaction whose rate constant is 0.2 sec^{-1} or longer could be determined. The sensitivity of the microcalorimeter, using 150 microliters of each reagent, is 10^{-4}Moles/L of a 1 Kilocal/mole reaction. About 4 experiments per hour can be performed. In order to greatly increase the thru put of the microcalorimeter, two approaches have been explored. One is a stopped-flow system which has proved effective for repeats of the same reaction, i.e., for kinetics 15 experiments per hour can be performed. Work is proceeding on the development of a system that will allow different reactions to be introduced at the same rate. The second method has been to immobolize an enzyme right on the surface of a thermistor. By immobilizing glucose oxidase and peroxidase, a usable system has been developed which in the presence of glucose and albumen, at pH 7.4, 25°C or 37°C gives excellent linearity from 5 mg % to 1000 mg % glucose. Further work is planned using whole blood. A new enzyme system for lactate determination is also being explored. Both of

these systems are being developed for in vivo use for the study of insulin action and the interrelation of lactate production and 2,3 Diphosphoglyceric acid metabolism in the red cell during exercise, particularly at the onset of anaerobic metabolism.

Proposed Research:

Development of the stopped-flow system will continue as well as the refinement of the simulation program for kinetics. Continued development of the enzyme thermistor sustem will be carried out with glucose, lactate, and 2-3 DPG. A simplified and improved thermistor will be built as a laboratory standard absolute temperature reference system for use in cuvettes.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01415-06 LTD

PERIOD COVERED
October 1, 1978 to September 30, 1979

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

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	R. Winslow	Clinician	CHB:NHLBI
Others:	M. Luzzana	Director Clinic Computer Center	U. of Milan
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	G. Dossi	Electrical Eng.	U. of Milan
	H. Cassio	Electrical Eng.	BEIB:DRS

COOPERATING UNITS (if any)
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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:
3	1.5	1.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The OECA(TM) has been modified somewhat to improve the temperature control. A new diffferential pH apparatus has been developed with a sensitivity of 0.1 millipH units. Preliminary tests indicate it has an excellent potential for enzyme determinations of Glucose, Lactate, and many other metabolytes and ions where a hydrogen ion is released or taken up as a part of the reaction. Many of these are needed for proper interpretation of the OEC.

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Project Description

Objectives:

The total oxygen needed by a normal subject is provided by the circulatory system accoridng to the well known equation:

O_2 consumption = cardiac output x arterial-venous O_2 difference.

Since several pathological conditions can shift the oxygen dissociation curve (ODC and thus how much oxygen can be released to the tissues, it is of consierable clinical and fundamental physiochemical interest to be able to measure the ODC under true physiological conditions of the patient. The aim of this project is to develop instrumentation to provide a comprehensive analysis of the various chemical factors regulating the (A-V) O_2 difference or, more generally, the oxygen dissociation curve of human blood under varous physiological or pathological conditions. ODC postion and shape is under control of various small molecules or ions, i.e, CO_2, protons, and 2,3-DPG, etc.

Methods Employed:

A systemic analysis of the complex interrlationship among several variables and thie effect on the oxygen dissociation curve requires the development of a simle method to obtain oxygen dissociation curves of human blood in vitro, under conditions closely simulating the in vivo situation to the patient.

Instruments are developed either at NIH and/or Milan, tests on pure hemoglobin are generally conducted first in Milan, where a large group is currently working on the purification of hemoglobin.

Testing on patient blood is then carried out in the Clinical Hematology Branch, NHLBI. Close cooperation exists with the medical school hospital in Milan where on-line computer monitoring will be carried out using the membrane oxygenator systm, developed in this laboratory by Dr. Kolobow.

Major Findings:

Small differences in temperatures have ben found to effect the shape of the ODC such that for 1% accuracy at least 0.02 °C stability is needed. By monitoring the temperature in the cuvette corrections can be made if necessary. A new improved cell was constructed with an

electrical temperature control. Stability and gradients appear to be better than 0.01°C across the cell which illiminates the need for corrections.

A differential pH apparatus has been constructed jointly with the Milan Group. Cell volume is 2 ml. Special 5 mm diameter glass electrodes with an electrolyte paste and gold shielding were constructed for us by Dr. W. Ingold, Inc., Zurich. A sensitivity of 0.1 pH unit was achieved after the differential pH meter developed in this laboratory was thermostatted. An improved temperature stability version is presently being built. Glucose determinations, as a simple enzyme test system, indicated good linearity from 20 to 600 mg % using a 10 ul sample and hexokinase. The use of a 2.5 mm diameter combintion electrodes made for us by Microelectrodes, Inc., New Hampshire, was also explored and proved to be less susceptable to junction potentials than the glass-glass system which uses a dialysis membrane as the liquid junction.

Program for Furutre Work:

The continued development of an OEC apparatus for high concentrations of hemoglobin by fuel cell methods and enzyme oxygen producing systems will be pursed. The OEC (TM) will be rebuilt to incorporate NIH developed electornics and produce an integrated package easy for the physicians technician to use. Work will continue on the differential pH apparatus with the testing of various chemical systems.

Publications:

1. Winslow, R. M., Morrissey, J. M., Berger, R. L., Smith, P. D. and Gibson, C.G.: Variablity of oxygen affinity of normal blood: An automated method of measurement, J. Applied. Phys. 45: 2890297, 19778.

2. Thiebault, L.E., Kusnetz, R., Winslow, R. M., and Berger, R. L.: An instrument to determine the hemoglobin oxygen equilibrium curve based on an analytical model for the transport of oxygen across a semi-permeable membrane. 1978 Advances in Bioengineering, p.107, American Society of Mechanical Engineers, New York. 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01418-04 LTD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Fast Responding Oxygen Electrode

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

P.I. : G. G. Vurek Senior Investigator LTD NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
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)

Work is in progress to develop a composite membrane covered oxygen electrode. The composite electrode should respond rapidly to oxygen partial pressure changes and simultaneously consume little oxygen from the sample.

845

PHS-6040
(Rev. 10-76)

Methods Employed:

A composite membrane made of porous metal or plastic filled with silicone rubber offers a way to control response time independently of effective gas permeability. Parameters of porosity, dimensions, and materials control both permeability and response time. The dominant factor for response time is dimension since diffusion down concentration gradients in the driving force for gas movement. Porosity and material composition control permeability. By arranging the geometry so that oxygen passing thrugh the membrane contacts the electrolyte at the cathode, the time required for passage through an electrolyte layer is minimized.

Major Findings:

Previous work with materials of porosity varying from 0.15% to more than 1% indicated that bulk porosity has a rather indirect influence on bulk permeability. Subsequent reevaluation of the data and actual geometry of the membranes suggest the optimum design consists of numerous short parallel holes filled with silicone rubber. The sample end of the holes is covered with a layer of silicone rubber. The other end of the holes is at the electrolyte. By making the holes in silver sheet, the silver can be used as the cathode. Some effort has been expended in obtaining a satisfactory electrolyte resistant bond between the silver and the silicone rubber.

Significance to Biomedical Research and the Program of the Institute:

Conventional oxygen electrodes consume substantial amounts of oxygen per unit area, leading to measurement errors caused by sample stirring or pressure induced dimensional changes. A common approach to the solution of this problem is to use either very small cathodes or thick membranes These cause substantial time lag problems or electrical measurement problems related to the small currents produced. The composite membrane approach offers a way to overcome these problems and at the same time providing a rugged, easily manufactured electrode.

Proposed Course:

A new silicone contact cement has been obtained. The supplier states it is very resistant to water and will provide excellent bonding of silicone membrane to the silver mesh we have been using. The silicone adhesive will fill the holes of the mesh, providing the difusion barrier needed as well as holding the outer silicone membrane. Response times and oxygen consumption of test composite structures will be measured.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01421-04 LTD

PERIOD COVERED

October 1, 1978 to September 30, 1979

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

P.I. :	R. Steele	Physical Scientist	LTD NHLBI
Others:	J. Handler	Section Schief	KE NHLBI
	J. Johnson	Medical Officer	Walter Reed
	M. Burg	Lab Chief	KE NHLBI
	G. Schwartz	Visiting Fellow	KE NHLBI

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

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Work has continued on methods and apparatus for the study of kidney and toad bladder epithelical cells grown as sheets on porous membranes. A device for sterile measurements of potential difference, and short circuit current has been made. It is useful for routine monitoring of cell preparations during growth. This design is possible because we grow the cells on membranes which are cemented to polycarbonate rings which provide electrical and chemcial isolation between the solutions contacting each side of the membrane.

A tonometer using silicone rubber tubing has been built for the study of the CO_2 to HCO_3^- pk in physiological salt solutions with and without 6% albumin. The pK of the albumin solution was measured to be 0.02 lower. This could result from the binding of 5% of the HCO_3^- to the albumin as occurs for Cl.

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1. Continue the development of methods and appartus for the study of kidney and toad bladder epithelial cells grown on sheets on porous membranes. Growth on opaque nucleopore membranes has been accomplihsed with great success. The current objectives are to find or make transparent membranes which are permeable to all the molecules in the tissue culture medium. This will allow microscopic observation of preparatrions prior to this electrophysiologic study. Since microscopic observation of cells is probably essential to the development of primary cultures, these transparent membranes will make it possible to grow primary cultures under conditions where nutrient and gas exchange is possible at both sides of the layer of cells. By this means, the conversion to anerobic metabolism found in many tissue culture cells grown on conventional petri dishes maybe avoided.

2. Develop a voltage clamping chamber (Ussing chambers) with very small solution volume and with gas exchanger for the simultaneous study of Na transport, O_2 uptake and CO_2 production by sheets of cells on pourous membranes.

3. Study the relationship between pCO_2, pH and total CO_2 in physiological salt solutions with and without albumin. Our recent results could be explained by the binding of 5% of the 25mM HCO_3^- to the 6% albumin present. HCO_3^- activity measurements with an HCO_3^- electrode recently developed by Simone and his associates in Switzerland should verify this interesting possiblity.

Methods Employed and Major Findings:

1. A voltage clamping chamber for sterile study of sheets of cells on a porous membrane was put into use. The use of membranes cemented to rings (1" I.D., 1-1/4" O.D. x 1/2" polycarbonate) which isolate the 2 sides of the membrane chemically and electrically is essential for the function of this device. An upper section slides on 3 rods so that it can be quickly raised while one of the cell sheet structure is placed into the lower section. The upper section carries 2 small calomel and cells, 2 small Ag-AgCl cells on the top side and 4 straight agar bridges (saturated KCl in Teflon tubing) extending out the bottom side. The bottom section has 2 channels which effectively extend 2 of the agar bridges to their proper position beneath the membrane which supports the sheet of cells. Sterile measurements are advantageous because optimum

growth period can be determined without destroying structures that are not yet ready. Also, the sterile measurement device allows short term measurements to be made very quickly.

Please see the Annual Report from Dr. J. Handler for a complete statement of the observation made on the cells. Breifly, one line of cells from toad bladder,TB-M, behaves much like the natural bladder. Another line, TB-6C, differs in that only the portion of its transport which was stimulated by aldosterone is blocked by amiloride. This agent completely blocks transport by the TB-M cells and natural bladders. Mitochondrial rich cells have not been found in either line of cells. These differences between cell lines and from natural bladders should prove to be useful tools in understanding the basic mechanims of active Na transport. However, continous changes in the cells and changes toward anerobic metabolism (indicated by increased lactate production compared with natural bladders) would certainly interfere with the study of this transport.

Some of the changes in these cells in culture and most other cells during tissue culture probably results from the impaired availability of nutrients and gas exchange which exists on the solid surface of a petri dish. Thus,it is expected that more normal cells and more types could be made to grow on membranes which are permeable to all of the constuents of tissue culture media and O_2 and CO_2. Primary culture techniques are greatly aided by microscopic observation. Thus such membranes should be tansparent. We have developed a collagen coating technique for a transparent regenerated cellulose membrane (0.020u to 0.035u pores) on which confluent sheets of toad kidney and toad bladder cells have grown. After some additional improvements, primary cultrues of toad bladder and Dr. Burg's rabbit kidney tubule cells will be tried. We have also made membranes of collagen (25u thick) which show promise. This maybe particularly useful for Dr. Spring's, LKEM, microsocpic studies which require a very thin membrane. They may also be useful for the membrane on very small rings in which just a few cells (maybe only one) can be grown out to confluence and studied electrophysiologically. This would be a unique approach to studying the function of the various cells in a heterogeneous population.

In the past I and Dr. Roy Maffly have studied the CO_2 production of toad bladders as function of active Na transport. Those preparations had 2 major disadvantages. (1) 80% of the natural bladders consists of connective tissue and muscle and contribute to the large (50%) nontransport CO_2 production. (2) The studies had to be done at pH 6.5 to minimize the CO_2 capacity of the Ringer's solution. The sheets of

toad bladder epithelial cells on membranes greatly reduces the amount of tissue which is not involved in transport. Also the great flatness of the membrane and high electrical resistance of the preparation should make it possible to design chambers with very thin layers of solution for the simultaneous measurement of short circuit current, O_2 consumption and CO_2 effux. The use of a gas exchange membrane in place of a carrier gas to separate the physiological solution from the CO_2 absorbtion solution (and O_2 delivery solution in this case) will help make this possible. Studies near pH 7.4 appear feasible.

Studies of perfused rabbit kidney tublue by Dr. Schwartz and Dr. Burg required knowledge of the effect of 6% albumin on the pK to the CO_2 to HCO_3^- reaction. To be able to measure the pK in physiological salt solution with and without the 6% albumin, a tonometer was made from 4 meters of silicone rubber tubing (1/8" dia. and 0.023" wall). A device to produce a back and forth movement of the solution in the tube of about 5 cm proved necessary to adequately stir the protein solution. The results showed by the pK of solution containing the albumin was 0.02 lower than the protein free solution. This could be explained by a 5% binding of the 25mM HCO_3^- to the 6% albumin. Such a HCO_3^- binding would be comparable to the known 5% binding of Cl^- 6% to albumin. Simone's group in Switzerland has developed a HCO_3^- electrode (they have augmented the HCO_3^- interference in one of their Cl^- electrodes as far as I can tell). The use of this electrode should help clarify this question of HCO_3^- binding to albumin.

Significance of Biomedical Research to the Program of the Institue:

The apparatus and methods necessary to grow and study sheets of cells on membranes will significantly advance the study of the basic mechanisms of active Na transport. The ability to make the measurements under sterile condition further aides this work. Dr. Handler, LKEM, NHLBI, has presented the physiological significance of this work in detail. The efforts at growing cells from primary culture on nutrient and gas permeable membranes may be a significant contribution to tissue culture technique in general in that the usual conversion to anerobic metabolism may be avoided. Also types of cells may be grown that were not possible before.

The study of HCO_3^- binding to albumin may improve our basic understanding of the relationship between pCO_2, pH and total CO_2 in blood serum.

Proposed Course:

1. Develope transparent membranes from the growth of sheets of cells which are permeable to nutrients, O_2 and CO_2.

2. Develope very small rings and membranes (i.e. collagan) for the growth of a few cells (even one) out to a confluent layer.

3. Develope apparatus for the simultaneous measurement of short circuit current, O_2 consumption and CO_2 efflux of sheets of cells on membranes.

4. Study the possible binding of HCO_3^- ions to albumin in physiological solutions.

Publications:

1. Handler, J. S., Steele, R. E., Sahib, M. K., Wade, J.B., Preston, A. S., Lawson, N.L., and Johnson, J. JH.: Toad urinary bladder epithelial cells in culture. Maintenance of epithelial structure, sodium transport, and response to hormones. Proceedings of Natl. Acad. Sci., U.S.A. (in press) 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01423-03 LTD

PERIOD COVERED
October 1, 1978 to September 30, 1979

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

P.I. :	R. L. Berger	Chief, Section on Biophys. Instrmentation	LTD NHLBI
	B. Balko	Expert Consultant	LTD NHLBI
	G. Liesegang	Staff Fellow	LTD NHLBI
	P. Smith	Expert Consultant	LTD NHLBI
Others:	T. Trayler	Professor of Chemistry	UCSD
	D. K. White	Postdoctoral Fellow	UCSD
	E. Bucci	Professor of Biochemistry	U of Md
	A. Schechter	Chief, Macromolecular Biology Section	LCB A

COOPERATING UNITS (if any)
BDTB Electrical Engineering Section
Department of Chemistry, UCSD

LAB/BRANCH
Laboratory of Technical Development

SECTION
Section on Biophysical Instrumentation

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 4	PROFESSIONAL: 2	OTHER: 2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Transmission and scattering (selective excitation double Mossbauer (SEDM)) spectrometers have been constructed and tested. The SEDM unit operates from 5° K to 330°K. Ferrochrome A has been examined by SEDM and found not to agree with the present theory regarding its energy exchange mechanisms. A quantum mechanical theory has been developed to interpret the transmission Mossbauer spectra line shapes. It is calculated by a program called SPIN 52. Computation of the SEDM line shapes taking into account an effective field approximation, arbitrary relaxation rates, and a thick scatterer is done using a program called SRELAX. A picosecond spectrometer is under construction to study protein dynamics. Primary wavelength is 1.06 microns using a Dye Mode Locked Nd-Glass Laser, amplification and doubling has been achieved as well as pulse selection by a Pockel cell.

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Project Description:

Objectives:

The objectives of this project are to develop new instrumentation, data handling techniques, mathematical methods, and models of the dynamics of energy exchange, conformational change and other dynamic phenomena involved in the reaction of proteins with their coenzymes and substrates. In particular to develop methods and instruments to study the local environment of the iron ion in hemoglobin under various conditions during oxygen binding to the hemoglobin tetramer and the α and β sites individually and to investiage differences between normal Hb, sickle cells (Hbs) and thalassemia blood.

Model reactions will be studied with especially prepared hematin compounds using replacement and pyrrole ring substituted compunds as well as naturally occurring variants in an attempt to understand the fundamental relations occurring in the hemoglobin reactions.

Methods Employed:

The methods employed are those of Mossbauer effect spectroscopy in the transmission scattering and selective excitation double Mossbauer (SEDM) modes and picosecond Laser spectroscopy. These involve cryogenic techniques which allow a range of sample temperatures of .05°k to 330°K.

Major Findings:

The program we have been developing for the study of protein dynamics has shown considerable progress this past year in both the selective Excitation Double Mossbauer (SEDM) and the picosecond spectrometer construction. The latter instrumentation is being developed to allow the investigation of events occurring on the picosecond time scale. The basis of this instrumentation is that lasers can be made to emit, under appropriate conditions, light pulses that have a duration of less than 10 picoseconds. Extensive study of these mode-locked lasers have been aimed at understanding their fundamental operation, but it is only recently that their application to the study of biological phenomena has been seen. For example, studies have been performed on the primary events of photosynthesis and vision, relaxation rates of proteins, and the role of hemeprotein structure on energy dissipation mechanisms. The development of this instrumentation will allow, with picosecond resolution, simultaneous

wavelength and time measurements. The particular system being developed will produce picosecond pulses at the following wavelengths: 1.06 microns, 530 nanometers, and 265 nanometers. In addition, a synchronous continuum, covering the visible spectrum, will be available as the interregation beam. The associated equipment for the detection, handling, and storage of the data are also being developed. At the present time, individual pulses at 1.06 microns and 530 nm have been generated.

The laser system was purchased in component form from Korad. It consists of a 6" x 1/2" rod as the oscillator and a 10" x 3/4" rod as the amplifier, (neodymium in phosphate glass). Mode-locking has been achieved using a saturable absorbing dye (Kodak 9860) as the passive mode-locker. Two configurations have been evaluated; first, a flowing dye cell in which the dye remains in contact with the 100% reflector, and second, a cell in which the dye could be positioned anywhere within the laser cavity. Of the two, the latter gives better and more reproducible mode-locking. The laser output consists of a pulse train of individual spikes separated by 7 nanoseconds; the total pulse train envelope varies between 100 and 500 nanoseconds. A pulse selector was purchased from Inrad. This consits of a pair of crossed calcite prisms between which is situated a KDP (potassium dihydrogen phosphate) Pockels cell. In the pre-firing position, a 7KV potential, corresponding to the half-wave voltage, is applied across the KDP crystal. A photo-trigger, derived from the laser beam, fires an avalanche diode array which rapidly switches the 7KV to ground potential through a Kryton tube. This corresponds optically to a gate, during which transmission through crossed polarisers is possible, of approximately 10 nanosecond. Single pulses of 10-20 mJ have been obtained. A substantial effort has been expended on trying to overcome the electrical interference produced by the fast switching of the high voltage. A shield has been constructed to totally enclose the pulse selector; preliminary testing shows that noise free operation of a photodetector can be obtained at the 1mV level with the shield rather than at the 100mV level without the shield. The coupling optics providing a 3:2 magnification of the beam between the oscillator and the amplifier have been aligned. The neodynium amplifier has been tested and its timing with respect to the oscillator set. A final setting of this delay is dependent upon being able to measure accurately the input and ouput pulses of the amplifier. The Pockel cell's shielding should now allow these mesurements to be made. Second harmonic generation, that is conversion of the 1.06 to 530nm, of the switched out pulse has been achieved using a Type IKDP crystal.

All components, except the echellon, are now in hand for the alignment of the photoactivating and detection light beams. A Princeton Applied Research Model 1216 vidicon detector and readout has been obtained and will be interfaced to a data collection system. A Digital Equipment Corporation MNC-11 system was chosen as the data collection system: it permits rapid acquisition and storage of the data on a 5 Megabyte hard disk, with individual experiments being transferred at a slower rate to Floppy disks.

Extensive mathematical work has been done during the last year in order to have an appropriate quantum mechanical theory to test the Mossbauer dynamics results. The scattering experiments on Ferrochrome A, a "textbook" model compound, have indicated that the present energy exchange relaxation model is not correct. A clear understanding of this processes is essential if we are to understand the energy utilization processes that occur when light is absorbed by a protein, or an energy exchange occurs between enzyme, coenzyme, and substrate which results in a dynamic conformational change occuring in the protein during the reaction. In general, it is this conformational change in the protein that makes possible the highly selective catalysis these compounds carry out. The effect of genetic and drug interaction on this process should prove of considerable fundamental interest in understanding these processes. On the experimental side both a transmission and scattering Mossbauer spectrometer are in operation with the capability of covering the temperature range of 5°K to 330°K. Because of the line splitting problems encountered with hemin and hemoglobin, a Helium dilution technique has been explored which showed that aplitting does occur, but only below 150 m°K. The ability to go to at least 50 millikelvin is presently being developed as only from the splitting can we understand the energy exchange mechanisms.

Proposed Future Research:

The picosecond spectrometer construction and testing will continue with operation expected by February 1, 1980. A high pressure cryogenic cell will be constructed for inclusion in the system as well as direct coupling to a PDP-11 computer. SEDM spectrometer development will continue with the construction of a Helium dilution cryostat for 50 millikelvin, or lower, operation. SEDM investigation of enriched hemin and model compunds will continue as well as hemoglobin chain and whole molecule experiments being started. The development of the appropriate mathematical theory to describe the SEDM line shapes and relaxation rates will continue with particular attention being paid to the ferrous state.

Publications:

1. B. Balko, E. V. Mielczarek: The Application of Selective Excitation Double Mossbauer to Time Dependent Effects in Biological Materials. Vol. 24, #1, 233, 1978.

2. B. Balko, E. V. Mielczarek, R. L. Berger: The Application of Selective Excitation Double Mossbauer Techniques to the Study of Relaxation in Ferrichrome A. J. de Physique, Colloque No. 2, Supplement #3, Tome 40, C2-17, 1979.

4. E. V. Mielczarek, B. Balko, R. L. Berger, D. K. White, T. G. Traylor, T. Mincey: Mossbauer Spectroscopy of the Model Hemoglobin Compound Dichelated Protoheme. J. de Physique, Colloque #2, Supplement #3, Tome 40, C2-495, 1979.

4. B. Balko, E. V. Mielczarek, R. L. Berger: Dynamics of the Local Iron Environment by the Selective Excitation. Double Mossbauer Technqiue (SEDM) in Frontiers of Biological Energetics from Electrons to Issues, Academic Press, N.Y. (1979(Vol I, pp. 617, Ed. D. L. Dutton, J. S. Leigh, A. Scarpa.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01424-03 LTD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

A Centrifuge for the Continuous Harvesting of Blood Cellular Components

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

PI:	T. Kolobow	Chief, Pulmonary & Cardiac Assist Devices	LTD NHLBI
	Y. Ito	Visiting Scientist	LTD NHLBI
	A. Pesenti	Visiting Fellow	LTD NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Technical Development

SECTION
Pulmonary and Cardiac Assist Devices

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

We have continued to improve on the design of the blood centrifuge previously developed in this laboratory. The blood separation chamber is a narrow ribbon like cell which can be either circular, spiral, helical, or a combination of the three.

Tubes leading to and from the continuous flow centrifuge are continuously untwisted using a previously described principle. Through proper placement of tubes, WBC, RBC, platelets and plasma can be continuously harvested. Improvements in the centrifuge design include a dynamic self balancing system, and special provision to exclude wear in the blood tubing during centrifugation.

At present, the centrifuge operates at a rotor speeds to 2500 RPM (400 G). Under those conditions, in excess of 100 ml/min of whole blood are continuously fractionated into blood and plasma components.

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Objectives:

To develop a continuous flow blood separator for long term and short term use, for the harvesting of plasma and blood cellular components for diagnostic and therapeutic purposes.

Methods Employed and Major Findings:

We are using a newly described principle for the continuous feeding of fluids into a rotating centrifuge whereby one avoids the use of rotating seals. A blood centrifuge using this principle is highly desirable because it avoids accidental contamination inherent in all rotating seal centrifuges.

We have designed a blood separator rotor where the separation chamber spirals towards the axis of the bowl in a narrow ribbon only - 1 1/2 mm. wide, 4.5 cm. high, and 60 cm. long. Blood enters the separation chambers at a point near the periphery and travels along the channel towards the center of the axis of rotation. Heavier blood cellular elements sediment in the G field towards the outer wall of the separation chamber, and are progressively displaced outward along the spiral toward the higher G-field; plasma and the lighter not yet sedimented cellular components, are displaced inward toward a lower G-field. The net result of the spiral configuration is that plasma (and unsedimented cells) flow in one direction, while sedimented cells flow in the opposite direction; the separated blood components can thus be harvested through properly placed pump lines.

The present principle of blood separation allows the detection of a sharp interface at all rotor speeds which is not possible in some competing blood separators.

The blood separation chamber can be made to conform/various geometrical designs. For instance, the separation chamber can be tilted by for instance 3^{o} from vertical to generate a spiral conical separation chamber, or a spiral-helical-conical separation chamber so as to impart different planes of separation to facilitate blood separation.

The centrifuge rotor is made of an aluminum plate, to which the desired channel geometry is imparted by welding appropriate ducts (or channels). A disposable silicone rubber insert is placed into these channels. No fasteners are needed because the hydraulic pressure generated by starting the centrifuge firmly anchors the separation chamber in place.

A number of improvements are incorporated in the present design. We had earlier attached a self-balancing mechanism to the centrifuge, which allowed for dynamic balancing of the rotor at speeds in excess of 600 RPM. We now use a multilumen tubing to replace individual tubes. We now also include means to prevent contact and wear at the two critical points where wear occurs from continued untwisting of the tubes.

The result of all this has been the elimination of the need to provide continuous, or intermittent lubrication to wear points along the blood fluid lines. Technically, this has been a major problem which may have posed safety considerations when used in humans.

At this point, we believe that all major technical problems in safe operation of this device have been overcome.

Significance to Biomedical Research and the Program of the Institute:

We foresee the use of the blood separator of above design to be useful for intermittent or continuous fractionation of blood cellular elements for research studies, for the harvesting of plasma, for plasma exchange, blood washing, extracorporeal irradiation of blood components, blood banking, for the pharmaceutical industry etc. Its major attributes are the lack of rotating seals and hence maintenance of sterility and cleanliness; and the engineering design to yield high efficiency blood separation.

As a bedside tool it is characterized by compact design, little blood trauma, and it can be operated with heparin as the only anticoagulant.

Proposed Course:

To continue studies on further optimization of this centrifuge.

Publications:

None

Honors and Awards:

Tech Ex. 1979 - Technical Excellence Award for Significant Achievement in Technological Development for Blood Cell Separator.
T. Kolobow, and Y. Ito., NHLBI.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 01428-02 LTD

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Countercurrent Chromatography with a New Horizontal Flow-through Planet Centrifuge

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

P.I.: Yoichiro Ito LTD NHLBI Medical Officer

COOPERATING UNITS (if any)

None

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SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

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1.0	1.0	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A versatile horizontal flow-through coil planet centrifuge has been introduced for performing countercurrent chromatography. The apparatus carries a pair of coiled separation columns. Both of these allow continuous elution simultaneously without the use of rotating seals. One column enables preparative-scale separations and the other, analytical-scale separations both with a high partition efficiency comparable to that obtained in liquid chromatography but without the complications arising from the use of solid supports. Capability of the appratus was demonstrated on separations of dinitrophenyl (DNP) amino acids and peptides using typical two-phase solvent systems.

860

Project Description:

Development of a new horizontal flow-through coil planet centrifuge for performing countercurrent chromatography for both preparative and analytical purposes.

Method Employed and Major Findings

a) Apparatus:

The design of the apparatus allows simultaneous elution through a pair of columns without the use of rotating seals. The rotary frame of the centrifuge is driven by the motor around the stationary pipe mounted horizontally on the central axis of the centrifuge. The rotary frame consists of a pair of rotary wings which hold a pair of column holders in the symmetrical positions 15 cm from the axis of the centrifuge. One holder called the pulley-side holder is equipped with a toothed pulley which is coupled with a toothed belt to an identical pulley mounted around the central stationary pipe. The coupling produces a planetary motion to the pulley-side holder; during one revolution of the rotary frame, the holder rotates once about its own axis in the opposite direction. The other holder called the gear-side holder is equipped with a toothed pulley which is coupled with a toothed belt to an identical idler pulley coaxially connected to an idler gear. The idler gear is again engaged to an identical stationary gear mounted around the central stationary pipe. This arrangement produces a planetary motion to the gear-side holder; during one revolution of the rotary frame, the holder rotates once about its own axis in the same direction. Combination of these two modes of planetary motion gives a great advantage in that the coiled column mounted on each holder can be eluted without the use of rotating seals.

The column is prepared by winding PTFE tubing onto a metal pipe to make a short column. The long column is made by connecting the desired number of column units, usually 10 short column units, in series in such a way that the tail end of a column unit joins to the head end of the next column unit. The typical column for large-scale separation is made of PTFE tubing 2.6 mm i.d. and 0.5 mm wall thickness wound around aluminum tubing with 1.25 cm o.d. and 1 mm wall thickness. The column for micro-scale separations is prepared from PTFE tubing, 0.55 mm i.d. and 0.3 mm wall thickness coiled onto stainless steel tubing with 0.68 cm o.d. and 1 mm wall thickness. The columns are symmetrically arranged around each column holder and counterweight pipes are mounted on the lighter side to balance the centrifuge.

Revolutional speed of the apparatus is continuously adjustable

from 0 to 600 rpm with high stability and accuracy with a motor control unit. A chromatronix cheminert pump was employed for elution and LKB Uvicord III for monitoring the absorbance at 280 nm.

b) Analysis of Acceleration Field:

Simple mathematical analysis was carried out to elucidate acceleration field acting on each column holder. The pulley-side holder is subjected to a uniformly circulating acceleration field acting in a plane perpendicular to the axis of the holder. The analysis also shows that at any given moment all locations on the holder experience the identical acceleration field. This indicates that the coiled columns can be mounted at any location on the holder to produce the same effects. This mode of acceleration field enables vigorous mixing of the two solvent phases in a narrow-bore tube to yield high efficiency analytical separations.

The gear-side holder is subjected to a complex pattern of acceleration field which undulates in both magnitude and acting direction during one revolutional cycle. Furthermore, the undulating pattern of the field changes with the location of the column on the holder. When the column is mounted coaxially close to the axis of the holder, the accelertion vector rotates around the column as on the pulley-side holder. When the column is mounted eccentrically remote from the axis of the holder, the undulating mode changes into a swinging motion. In this case the two immiscible solvent phases in the coiled tube are separated by the centrifugal force in such a way that the heavier phase occupies the outer portion and the lighter phase, the inner portion of each coil unit, each phase segment swinging back and forth with revolution to accelerate partition process. This gives stable retention of the stationary phase in a large-bore column for preparative-scale separations.

c) Studies on Retention of the Stationary Phase:

Retention of the stationary phase in a large-bore preparative column was studied on a short column prepared from 2.6 mm i.d. PTFE tubing coiled around a 1.25 cm o.d. core to make 100 coil units with a total capacity of about 26 ml. The column was mounted on each holder at a location 3.5 cm from the axis of the holder. The following two-phase solvent systems with a variety of interfacial tension were selected: n-BuOH/CH_3COOH/H_2O (4:1:5), $CHCl_3$/CH_3COOH/H_2O (2:2:1), Ethylacetate/10% CH_3COOH, 5% NaCl aqueous solution (1:1), and Hexane/H_2O. The retention of the stationary phase measured was expressed as retention % (stationary phase volume retained in the column X 100/total column space) and plotted against the applied revolutional

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speed to obtain a retention curve for each solvent system. The flow rates of 12, 24, 60, and 120 ml/hr were tested. Retention curves obtained from the pulley-side column display a great variety in shape according to interfacial tension and viscosity of the two-phase solvent systems. For a low interfcial tension, high viscosity n-BuOH system, retention % declined sharply with the revolutional speed where stroboscopic observation revealed intensive emulsification of the phases in the coiled column. For other phase systems, retention curves follow a characteristic pattern with the rate of revolution. At a low revolutional speed around 200 rpm, the curvces of non-aqueous stationary phases exhibit a sharp rise which becomes more pronounced in the solvent systems with higher interfacial tension. After this critical range of rpm, the retention becomes stable but the retention levels for the non-aqueous phase always exceed those for the aqueous phase in the same solvent system. On the other hand, the retention curves for the gear-side column follow a quite different pattern. In all solvent systems the curves smoothly approach the stable levels at a relatively low rpm and then display a long plateau up to the maximum revolutional speed of 600 rpm. These retention levels at the plateau are mostly within an ideal range for both non-aqueous and aqueous stationary phases. Stroboscopic observation of the gear-side column reveals complete separation of the two phases in each turn of the coil with undulating interfaces swinging back and forth during each cycle of revolution. This finding is consistent with the results of analysis on the acceleration field described earlier. Overall results indicate that for the use of a large-bore column the gear-side holder has greater advantages over the pulley- side holder in that satisfactory retention is available for a broad spectrum of solvent systems under a wide range of revolutional speeds and flow rates. Retention of the stationary phase in a small-bore column was studied on a short column prepared from 0.55 mm i.d. PTFE tubing coiled onto a 0.68 cm o.d. core making approximately 340 helical turns with a total capacity of about 2.4 ml. The column was mounted on each holder at a location 2 cm from the axis of the holder. Using flow rates of 6 and 2.4 ml/hr, retention curves were obtained from the n-BuOH/CH_3COOH/H_2O (4:1:5) and $CHCl_3$/CH_3COOH/0.1N HCl (2:2:1). The overall results revealed characteristic features of the gear-side and pulley-side columns observed in the previous studies on the large-bore column. Compared with the previous data, the retention of the aqueous phases is much less than that of the non-aqueous phases especially in pulley-side column. This result suggests that in the small-bore column the effects of solvent-wall interaction on retention become more significant and the retention strongly favors the non-aqueous phase which has an affinity to

the PTFE tube. In the n-BuOH phase group, the pulley-side provides satisfactory retention for the non-aqueous phases while retention for the aqueous phase requires a greater centrifugal force field to reach a suitable level.

d) Studies on Partition Efficiency:

Partition efficiencies of both large-bore columns were examined with the short columns previously used for retention studies. The two typical phase systems were selected for separation of samples with suitable partition coefficients, i.e., $CHCl_3/CH_3COOH/0.1N$ HCl (2:2:1) for separation of DNP-DL-glutamic acid and DNP-L-alanine and n-BuOH/CH_3COOH/H_2O (4:1:5) for separation of L-valyl-L-tyrosine and L-tryptophyl-L-tyrosine. In each separation, the column was first filled with the stationary phase followed by the sample injection through the sample port. Then the mobile phase was eluted at a given flow rate while the apparatus was spun at a desired revolutional speed. The eluate was continuously monitored through an LKB Uvicord III at 280 nm. Th efficiency of the large-bore column obtained from the gear-side column sharply increases with the revolutional speed up to 400 rpm. The pulley-side column gives quite different results. The efficiency becomes maximum at relatively slow revolutional speeds of around 100 to 200 rpm where further increase of the revolutional speed results in a sharp decrease of the peak resolution. Also, in the pulley-side column, the results obtained by the stationary non-aqueous phase are much better than those obtained by the stationary aqueous phase, while the choice of the stationary phase makes little difference in resolution for the gear-side column. These results clearly indicate that the gear-side column is much superior to the pulley-side column when a large-bore coiled tube is used. Partition efficiency of the small-bore column showed different results in that the pulley-side column yielded substantially higher efficiency than that in the gear-side column for the chloroform phase system.

e) Countercurrent Chromatography with Long Coiled Columns:

Highly efficient separations were achieved with both large-bore and small-bore columns, each equivalent to 10 column units applied in the previous studies. The large-bore column was mounted on the gear-side holder and the small-bore column, on the pulley-side holder. The chloroform phase system was used for separation of a set of DNP amino acids and the n-BuOH phase system for separation of oligopeptides. The two-phase solvent systems were thoroughly equilibrated in a separatory funnel at room temperature and separated before use. Sample solutions were prepared by dissolving a set of samples in the stationary phase and these were stored in the dark at 4°C. In each experiment the column

was first filled with the stationary phase followed by sample injection through the sample port. Then the mobile phase was eluted at the optimum flow rate while the apparatus was spun at the optimum revolutional speed determined by the previous experiments. The eluate was continuously monitored with an LKB Uvicord III at 280 nm through a 1.8 mm light path flow cell. All separations were successfully performd yielding high partition efficiencies of 1000 theoretical plates for the large-bore column and 4000 theoretical plates for the small-bore column as predicted by the results of efficiency studies using a short column. The method has been applied to separation of synthetic polypeptides with 20 amino acid residues, gramicidins and prostaglandins with promising results.

Significance to Biomedical Research and the Program of the Institute:

Countercurrent chromatography utilizes no solid support and therefore eliminates all complications arising from the use of solid supports. Consequently, the method gives a high recovery of samples and yields high purity fractions with high reproducibility and predictability. The present method will be useful for separations and purification of various biological materials.

Proposed Course:

Application to separation of various biological materials.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01429-02 LTD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Ultrasensitive Nitrogen and Ammonia Gas Measurement

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

P.I. : G. G. Vurek 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:
1/10	1/10	

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)

This project concerns the development of luminescence methods for the measurement of very small amounts of gaseous nitrogen and ammonia gas. These methods may have applicability to the measurement of the partial pressure of nitrogen in blood and to the determination of ultra-micro amounts of ammonia. Emission of characteristic spectral bands by both nitrogen and ammonia can be excited by passing the gas through a radio-frequency electric discharge. In addition, chemiluminescent reactions can be exploited. Nitrogen flows of 5×10^{-11} 1/sec can be measured.

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PHS-6040
(Rev. 10-76)

Objectives:

The objective of this study is to develop methods suitable for rapid and convenient measurement of gaseous nitrogen and ammonia. Blood nitrogen partial pressure reflects the effective ventilation of various regions of the lung. Methods used previously have relied on gas chromatography or mass spectroscopy. The luminescence method may offer greater convenience and simplicity. In addition, the development of a simple method for picomole amounts of ammonia would be of use to researchers studying metabolic processes.

Methods Employed:

Two techniques are being explored presently. One uses a radio-frequency electric discharge and the second is based on chemiluminescent processes. In the former, ultrapure helium carries the nitrogen to be mesured into an inductively-coupled R-F discharge. At pressures of a few hundred pascals, specific nitrogen emission bands are produced by the discharge. The intensity of the bands is proportional to the amount of nitrogen present. Ammonia gas can be measured this way, too. In addition to the RF discharge technique for ammonia there are sensitive chemiluminescent methods. One relies on the conversion of ammonia to nitric oxide which can react with ozone to produce light. Another is based on the direct light releasing reaction of ammonia with optically pumped metastable mercury. These chemiluminescent approaches have the potential of greater sensitivity than the electric discharge technique.

Major Findings:

During the time avilable for this project, considerable effort was made to improve the performance of the R-F discharge system. Two areas received the major effort: background reducton and response time improvement. Because the ultimate sensitivity of the system depends on the amount of nitrogen in the carrier gas, purification of the carrier is important. In our system, liquid-nitrogen cooled molecular sieve traps impurities entering the system from the helium tank and associated plumbing. Another trap placed downstream from the discharge chamber prevents backstreaming of impurities from the vacuum pump. The ultimate limit is determined by the background continuous emission of the helium discharge. Present results indicate the background corresponds to less than 50 picoliters of nitrogen per second. The response time has been reduced to approximately 15 seconds (63% of final value).

This may be adequate for the measurement of blood PN_2 but greater sensitivity is neeeded for the microammonia technique. For that reason, preliminary studies of chemiluminescent techniques for ammonia are now under way.

Significance to Biomedical Research and the Program of the Institute:

The development of these luminescent methods may provide simpler and more economic approaches to the mesurement of blood PN_2 and micro-amounts of ammonia. Use of blood PN_2 measurement may facilitate pulmonary function studies and improve respiratory care. The microammonia technqiue may facilitate renal research.

Proposed Course:

The RF discharge method may now have sufficient sensitivity to begin testing the influence of other gases (water,vapor, oxygen, carbon dioxide) on the system. Follow the results of those studies, simulates blood PN_2 measurements will be undertaken. Further evaluation of chemiluminescent technqiues for ammonia will be undertaken.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01431-01 LTD

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (30 characters or less)

Cell Separation with Physiologoical Solution with the Non-Synchronous Flow-Through Coil Planet Centrifuge

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

P.I.:	Yoichiro Ito	Medical Officer	LTD NHLBI
	Peter Carmeci	Electronic Engineer	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:
1.5	1.5	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A new cell elutriation technique uses a slowly rotating coiled tube in a centrifugal force field. Nonsynchronous flow-through coil planet centrifuge enables a continuous elution with a physiological saline solution through the rotating coiled column. Capability of the method was demonstrated on separation of human and sheep erythrocytes.

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Project Description

Objectives:

Separation of cells with a physiological saline solution by the non-synchronous flow-through coil planet centrifuge.

Method Employed and Major Findings:

1) Principle:

Let us consider a liquid-filled coiled tube slowly rotating around its horizontally oriented axis in the gravitational acceleration field to study the motion of the particles suspended in the liquid. If the particles are either lighter or heavier than the liquid, rotation of the coil forces these particles to move toward one end of the coil. This end of the coil is called the head and the other end, the tail. When the rotational speed of the coil is relatively slow, the particles lighter than the liquid always stay on the top of the coil while the particles heavier than the liquid always remain at the bottom of the coil, both moving toward the head of the coil at a rate of one helical turn per one rotation of the coil. Therefore, no separation is observed in this case. As the rotational speed of the coil is increased, particles with small sedimentation velocities fail to maintain the same position in the coil with respect to the gravity and are retarded in their movement to the next coil unit and therefore proceed toward the head end at a slower rate. this retardation results in separation of these particles along the coiled tube. As the rotational speed is further increased, this retardation takes place for all particles but the degree of retardation depends upon the sedimentation rates of the particles. Consequently, over a given period of time, the particles are separated by their sedimentation rates in a spectrum along the coiled tube with the particles that have large sedimentation rates being closer to the head end and those with smaller sedimentation rates closer to the trial.

In order to facilitate fractionation of the separated samples, introduction of a flow-through mechanism is desired. The nonsynchronous flow-through coil planet centrifuge described earlier (Z01 HL 01427-01 LTD) enables continuous flow through the slowly rotating coiled tube from the head end to elute the cells through the tail end while the centrifugal force accelerates the sedimentation of cell particles. Thus the particles with greater sedimentation rates are retained in the coiled tube for a longer period of time whereas particles with smaller sedimentation rates are eluted earlier. Consequently, the particles are continuously fractionated in the order of their relative sedimentation rates.

2) Method:

The separation column was prepared from a 6 m long, 1 mm i.d. PTFE tube coiled onto 6 mm o.d. cores, making about 200 helical turns with a total capacity of 5.2 ml. Isotonic buffered saline solution (Ph 7.4) was prepared by dissolving NaCl 90 g, Na_2HPO_4 13.65g, and $NaH_2PO_4 \cdot H_2O$ 2.15g in 1 liter of distilled water and diluting 85 ml of this stock solution with distilled water to bring the final volume to 1 liter. Erythrocyte samples were prepared from EDTA-treated fresh blood by washing with the buffered saline solution three times, repeating centrifugation and decanting the supernatant. The obtained packed cells were finally suspended in the same solution at a concentration of Hct 20%. Equal amounts of sheep and human cell suspensions were mixed and a volume of 0.2 ml was used for each separation. The coiled column was first filled with the buffered saline solution and the sample suspension was then introduced through the sample port. This was followed by elution with the same solution at 3.6 ml/hr while the apparatus was spun at 520 rpm (50g) with 3.6 rpm for coil rotation. Fractionated samples (1.2 ml/tube) were analyzed microscopically with a blood-counting chamber and also with a Coulter size-distribution apparatus (Model B).

3) Results:

Human and sheep erythrocytes were separated into two peaks. The first peak consisted of sheep cells and the second peak, human erythrocytes. The overall patttern of the eluted erythrocytes closely resembles the size-distribution curve of the original sample mixture obtained with Coulter analyzer. The human cells showed a broader distribution than the sheep cells. The analysis of human cell fractions clearly indicates that the cell size increases with the fraction number and some human cells that still remained in the coiled tube were the greatest in size. Microscopically the cells were well preserved and no visual evidence of hemolysis was observed during separation.

Significance to Biomedical Research and the Program of the Institute:

Most conventional cell separation techniques utilize non-physiological media which may alter the physiological function of cells. In the present method cells are only exposed to physiological solution for relatively short period of time, which will preserve normal cell function. Therefore, the method wil be useful for cell separation in biomedial research laboratories.

Proposed Course:

1. Separation of other types of cells.

2. Development of a rotating-seal-free nonsynchronous coil planet centrifuge.

Publications:

Ito, Y., Carmeci, P., and Sutherland, I. A.: Nonsynchronous Flow-Through Coil Planet Centrifuge Applied to Cell Separation with Physiological Solution. Analytical Biochemistry, 94, 249-252, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01433-01 LTD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Feasibility Studies in Hybrid Artificial Internal Organs

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

P. I. :	T. Kolobow	Section on Pulmonary & Cardiac Assist Devices	LTD NHLBI
Others :	E. A. Jones	Acting Chief, Section on Diseaes of the Liver	DD:A
	M. I. Zeneroli	Visiting Scientist	DD:A

COOPERATING UNITS (if any)

Digestive Diseases Branch, NIAMDD

LAB/BRANCH
Laboratory of Technical Development

SECTION
Section on Pulmonary and Cardiac Assist Devices

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

We have devised a hybrid artificial internal organ (such as the hybrid artificial liver), where a suspension of cells and tissue fragments (such as from the liver) is kept in contact with a microporous membrane, and the other side of which is exposed to flowing fresh blood from a recipient. The overall configuration fo the reactor is similar to the spiral coiled membrane lung, except that the suspension of cells - tissue fragments is replacing the gas phase in the membrane lung. The biochemical activity of cells thus continues, and such a reactor can thereby supplement the failing organ of a patient.

PHS-6040
(Rev. 10-76)

Objectives:

To exploit the biochemical function of homologous and heterologous tissue as a biological reactor.

Methods Employed and Major Findings:

Previous work in this laboratory has shown that whole blood under all conditions studied is the best perfusate at near body temperature. We have performed these studies on the excised lamb heart, where changes in perfusate flow or the quality of the perfusate could be immediately assessed through change in mechanical performance of the heart.

Recent work in this laboratory has also shown that organs chopped into small fragments can function, and grow in mass for weeks when separated in an ex vivo system by a microporous membrane from flowing fresh blood. This implied that the membrane provided for sufficient nutrient and metabolite and hormonal exchange from blood; it also showed that the donor cells were not attacked by the host and destroyed.

Over the years, there have been many attempts to utilize whole donor organs to supplement a failing organ in man. Some success has been reported in man using the pig liver, although only for short term. Hence this interest to devise a hybrid artificial internal organ.

From the above studies we conclude it possible to devise a reactor consisting of a suspension of cells from a whole organ (such as sheep liver) separated by a microporous membrane. This reactor can then be connected in an ex vivo system to a patient who acutely may need a support system.

We have devised the framework of the hybrid organ of microporous polypropylene membrane, made into a thin envelope, and wrapped it around a central spool. Overall, its configuration is similar to the spiral coiled membrane lung except that the gas phase is replaced by a suspension of finely devided liver, adrenal, etc.

We consider it most important to devise a method to rapidly harvest a suspension of finely divided organ, perhaps of the order of 500 to 1000 gm of tissue. We have devised a pneumatic system where a whole organ is pressed through a series of screens of various meshes and provide us in a few minutes a relatively uniform suspension of organ fragments. We have introduced this suspension into the reactor system where it was uniformly distributed over the surface of the membrane.

Significance to Biomedical Research and the Program of the Institute:

Artificial internal organs, such as the artificial kidney machine, are passive reactors, without biological control.

On the other hand, the use of living cells or tissue separated by a micro-

porous membrane can satisfy different requirements, such as active biological synthesis as found in the intact organ. Such a reactor may be particularly useful in acute, or chronic hepatic insufficiency. Similarly, tumor cells or tumor aggregates may be grown in such a reactor in a native environment and response to treatment dose and schedule can be assessed on a small scale, the system being connected ex vivo to the native donor. (i.e. the patient who has cancer). The overall attractiveness of the hybrid organ (organ culture, tumor culture) lies in simplicity of use, and the isolation of cells by a microporous membrane.

Proposed Course:

To optimize technical aspects of the design, and to prove the concept using various tissues.

Publications:

1. Zeneroli, M.I., Waggoner, J.C., Shhafer, D.F., Kolobow, T., Jones, E.A.: Development of an extracorporeal hepatic support device: clearance of amino acids, short chain fatty acids, and neurotransmitters by different carbons. Proceedings of the Rome Congress on Amino Acids and Hepatic Failure. In Press., 1979.

875

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01434-01 LTD

PERIOD COVERED
October 1, 1978 to September 30, 1979

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

P.I. : G. G. Vurek Senior Investigator LTD NHLBI

COOPERATING UNITS (if any)

LKEM, NHLBI

LAB/BRANCH

Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2/3	2/3	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Measurement of picomole amounts of magnesium ion by colorimetry can be accomplished with the aid of a new microcolorimeter. The working volume of the colorimeter is less than 200 nl. High precision light transmission measurements are facilitated using digital techniques.

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Objectives:

The objective of this project is to explore new designs for microcolorimeter, using the measurement of picomole amounts of magnesium as a test system.

Methods Employed:

We use a standard colorimetric test system for magnesium ions having good sensitivity and specificity for magnesium in the range of sample concentrations of 0-1 mM. Chelating and complexing reagents eliminate interference from other ions. The absorption maximum is near 630nm which is close to the wavelength emitted by He-Ne lasers and some light emitting diods. Various colorimeter combinations using lasers, LED's, and conventional light sources have been explored for ease of sample handling and overall performance. Fiber optic devices were also explored as means for putting light into and passing it out of the sample holder. Solid state light detectors operate well at that wavelength region. A photodioide, operated in the photovoltaic mode, is used to measure light transmitted through the sample. The output of the operational amplifier, which converts the photosignal to a voltage, feeds a voltage-to-frequency converter. The frequency generated can be counted easily. In addition, a second photo detector system can be used to generate a reference signal so that a simple ratio scheme can be achieved.

Major Findings:

Several configurations of the microcolorimeter designed earlier which used fiber optics ere explored. These configurations were based on the requirement for a flow-through device, for these are numerous applications in separation science for a submicroliter flow through calorimeter, as well as the meassurement of discrete samples of, for example, magnesium ions. None of the configurations involving long (10 mm) paths were sufficiently rugged or free from geometrical problems. The current design uses a capillary tube, 0.5 mm I.D. passed through the beam from a filtered incandescent lamp. Using the digital techniues mentioned above, the sensitivity of the method is about 2×10^{-13} mol of magnesium. After exploring numerous colorimetric procedures for magnesium measurement, we chose a method based on a dye, "Calmagite", which has been incorporated into a commercial clinical chemistry list. This kit provides good sensitivity and specificity; however, the color change seems to be unstable if the reaction product is exposed to air for prolonged periods. This color shift may be due to a pH shift caused by absorption of CO_2 from the air. The color shift occurs too slowly to be significant- more than 6 hours are required for observable change.

Significance to Biomedical Research and the Program of the Institute:

The new colorimeter has applications to various physiologic studies because numerous material of interest can be measured colorimetrically. In addition, separtion systems such as Dr. Ito's microscale countercurrent chromatograph and other liquid chromatographs can utilize a high sensitivity monitor of the sort developed here.

Proposed Course:

Further work to establish the best configuration and sample handling techniques will be performed and the full method for micro-magnesium measurements will be worked out.

Publications:

None

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01435-01 LTD

PERIOD COVERED

October 1, 1978 to September 30, 1979

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

P.I. :	R. L. Bowman	Chief, Lab. Technical Dev.	LTD NHLBI
Others:	D. Bauk	Guest Worker	LRD NHLBI

COOPERATING UNITS (if any)

Diagnostic Radiology Department, Clinical Center

LAB/BRANCH

Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Current devices for access to specific pathological lesion for radiologic intervention via the vascular system under radiological guidance require more control and versatility. The topocatheter guidance system which was demonstrated several years ago is not in use because of difficulties in construction and use. The present system has shortened the length requred, provided a taper to reduce the self interference, provided access to the lumen without a long guide and shortens the length of guiding filaments and permits control without running them thru the seal area. The purpose of this work is to demonstrate a configuration that can be used to guide the catheter for access to the vascular system.

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Project Description

Objective:

To provide a catheter tip that can be directed into specific blood vessels or ducts as it is advanced under radiologic observation. A system demonstrated (1976) was not implemented because engineering technology could not provide the design in the small sizes required. An alternate to the thin walls by a special grooved design without the guidance fiber has control limitations and requires an inconvenient long external tube to accommodate the unextended catheter.

Methods Employed:

The new concept retains the fiber guidance system but places only a short section of the topocatheter with its frictionless "rolling advance" and directional control on the end of a conventional catheter. A 3mm o.d. catheter is fitted with a tapered section of "topocatheter" which is contained within the conventional catheter until advanced by hydrostatic pressure. Control fibers run over the outside of the extendable portion and return via the lumen of both portions of the catheter. The portion attached to the conventional catheter is 3mm in diameter and tapers to 1 mm diameter when fully extended. The extendable portion is a double walled tube with lubricating, radiopaque fluid between the walls, a slight constriction at the tip of the conventional catheter divides the double walled section into interior and exterior compartments so that a pulse of increased pressure will cause the internal compartment to be reduced as the external compartment fills thus causing the inner wall to move out of the holding catheter and roll its evertingg surface into the vessel without sliding friction until its smallest diameter reaches the total extension. The tapered form provides freedom from interference from the inner wall against the outer wall because of the smaller diameter. The radiopaque hydraulic fluid acting as a lubricant between the walls also helps to provide x-ray density for visualizing the tip position. Fine aramid fibers provide guidance by controling the unrolling of the everting portion one or more fibers can be used to direct the tip as it everts. A model system was made by casting urethane in dimethylformauricil on to a form made of two tapered metal pieces that screwed together at their narrow tips. The cured casting was removed by unscrewing the tips and pulling out the forms from each end. A 10 cm catheter was then made by everting both ends of this casting and attaching them to an annular section that provided a fine hole for filling the space between the walls. This annular section provides the hydrostatic construction and a point of attachement to the standard catheter.

Major Findings:

In the 1 to 3 mm taper model the thin wall urethane tips were demonstrated to advance and also retract with of hydrostatic pressure applied to the holding catheter. No directional control or actual use in turning corners was performed but the simplicity of adding the fibers is obvious and previously this method proved very effective. The elimination of the long container for access to the lumen is an additional convenience. Strength and dependability of the thin films has not been attained but the crude dip coats used was able to demonstrate the principle.

Significance to Biomedical Research and the Program of the Institute:

Interventional radiology has been demonstrated to be an effective alternative to surgical access to therapeutic closure or opening of vascular channels. Aneurysms, fistulae, constrictions and tumor circulations have been treated but access to small vessels and difficult to enter branches still limits the procedure.

The rolling wall or topocatheter is an old idea but the addition of the control and reduction of wall interference may provide the additional versatility to use of the method.

Proposed Course:

To effectivly demonstrate that these devices can be made in the very small sizes required.

Publications:

J. L. Doppman, W. Aven, R. L. Bwoman, L. L. Wood, and M. Girton: A Rapidly Polymerizing Polyurethane for Transcatheter Embolization. Cardiovasc. Radiol. 1, 109-116, 1978.

ANNUAL REPORT OF THE
SECTION ON THEORETICAL BIOPHYSICS
OFFICE OF THE DIRECTOR OF INTRAMURAL RESEARCH
NATIONAL HEART, LUNG, & BLOOD INSTITUTE
October 1, 1978 through September 30, 1979

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.

Specifically: Work has continued on the qualitative analysis of renal flow networks. Earlier results for a system of differential equations for a counterflow system of parallel flow tubes have been extended to include an existence theorem that allows some tubes with positive diffusion and other tubes with zero diffusion. Earlier work for a single tube exchanging with an interstitium has been extended to show existence and uniqueness for variable pressure. As pointed out last year, for certain parameter choices, the three tube central core model shows multiple solutions. Study of the behavior of these solutions as a function of inlet flow and tubal transport properties has continued.

In computer simulation of the mammalian kidney, considerable attention was directed to improved numerical techniques for the solution of large systems of differential equations. Thus, the analytic expression of the pointwise Jacobian was extended to include a system of six equations in six unknowns. This permits four solutes, hydraulic pressure, and volume flow to be included as tubal variables. In solving the tubal equations the amount of calculation has been reduced by a factor of three. This scheme has been incorporated into the multinephron full kidney model, a six-tube vasa recta model, and a central core model. For the interstitial and boundary equations, a numerical Jacobian must still be calculated. A rank one update of the inverse of this Jacobian matrix has substantially reduced the calculation required. This scheme has been incorporated into the full kidney multinephron model, a multinephron central core model and a single nephron vasa recta model. Storage requirements and calculation have also been reduced by the incorporation of selected subroutines for matrix manipulation from LINPACK. In collaboration with R. P. Tewarson (SUNY) the possible utilization of cubic and quintic splines has been explored.

The improved methods have been used to verify earlier calculations. No significant differences have been found. New studies have been undertaken on the role of urea cycling in the concentration of urine. Studies on the functional implication of renal architecture have continued with the implementation of a model with direct solute and water exchange between the vasculature, the ascending limb of Henle and the interstitial space.

In the epithelial studies, attention has been focused on the representation of solute linked water transport in a leaky epithelium. The analytical model of the lateral intercellular space has been developed and used to identify the determinants of isotonic transport, to estimate the influence of intraepithelial solute polarization on the measurement of epithelial water permeability, and to discuss the capability of interspace models to simulate transport of water against an osmotic gradient. For a compliant interspace, the analytical model has been used to isolate the time constants of intraepithelial transients in a form suitable for experimental investigation. Analytical predictions have been verified against the more complete numerical epithelial models.

A collaborative project with the Laboratory of Kidney and Electrolyte Metabolism has involved the simulation of passive transport of CO_2 across proximal tubule in an isolated perfused tubule preparation. The effect of intratubular buffering on the steady state CO_2 concentration profile has been estimated analytically. A numerical model that defines the collected fluid pH as a function of perfusion conditions and tubule permeability has been used in the reduction of experimental data.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03201-19 STB

PERIOD COVERED

October 1, 1978 through September 30, 1979

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:
.8	.6	.2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The purpose of this project is to develop the general theory of transport and flow processes taking place in the kidney. Aims of current work include (1) Thermodynamic 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.

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PHS-6040
(Rev. 10-76)

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 qualitative analysis of equations describing kidney models, and the development of analytical solutions of kidney models.

During the past year, work has continued on the qualitative analysis of renal flow networks. Earlier results for a system of differential equations for a counterflow system of n parallel flow tubes has been extended to include an existence theorem that allows, in the model, some tubes with positive diffusion and other tubes with zero diffusion. A report on the earlier work of a problem for a single tube exchanging with an interstitium has been accepted for publication.

The results for the one tube problem have been extended to include variable pressure. Letting $C(x)$, $F(x)$, and $p(x)$ denote respectively the solute concentration, flow rate, and pressure, in the tube, and letting primes denote differentiation with respect to x, the equations are

$$- DC'' + (FC)' + J_s(x,C,p) = 0$$

$$F' + J_v(x,C,p) = 0$$

$$p' + RF = 0$$

and the boundary conditions are

$$C(0) = C_0 > 0$$
$$C(1) = C_1 > 0$$
$$p(0) = p_0$$
$$p(1) = p_1.$$

Existence and uniqueness results have been obtained by making certain assumptions on the functions J_s and J_v. The existence theorem was obtained by using a fixed point argument and the uniqueness was obtained by making an analysis of the linearized problem.

A mathematical analysis, including existence and uniqueness, was made for some boundary value problems which model the flow of a fluid-solute mixture in a system of two interacting tubes which are connected at one end. The differential equations are of the type

$$-D_i C_i'' + (F_i C_i)' = -J_{si}(x,C_1,C_2)$$

$$F_i' = -J_{vi}(x,C_1,C_2) , \quad i = 1,2.$$

When $D_1 > 0$, $D_2 > 0$, the boundary conditions are

$$C_1(0) = C_{10} > 0$$

$$C_2(0) = C_{20} > 0$$

$$C_1(1) = C_2(1)$$

$$DC_1'(1) = -D_2C_2'(1)$$

$$F_1(0) = F_0$$

$$F_1(1) = -F_2(1).$$

When $D_1 = D_2 = 0$, the boundary conditions are either

$$C_1(0) = C_{10} > 0$$

$$C_1(1) = C_2(1)$$

$$F_1(0) = F_0$$

$$F_1(1) = -F_2(1)$$

or

$$C_2(0) = C_{20} > 0$$

$$C_1(1) = C_2(1)$$

$$F_1(0) = F_0$$

$$F_1(1) = -F_2(1).$$

A fixed point argument was used to obtain existence. In the case $D_1 = D_2 = 0$ a uniqueness criteria was obtained by reducing a boundary value problem to an initial value problem. A nonexistence result was also obtained that demonstrates the need for $|F_0|$ to be sufficiently large for existence to follow.

The analysis and numerical study of the three tube central core model of the renal medulla, as defined in last year's report, has continued. This study demonstrates the existence of several solutions to the equations of the model, and discusses their behavior as a function of inlet flow and water permeability.

Proposed Course:

We plan to continue work on the general qualitative analysis of the kidney equations. In the immediate future we plan to concentrate on the

problem of non-unique solutions that have been demonstrated for particular central core models.

Publications:

Garner, J. B., and Kellogg, R. B., A one tube flow problem arising in physiology. Bull. of Math. Biology (in press).

Stephenson, J. L. Report on mathematical models of the kidney and its subsystems, to the subcommittee on normal kidney structure and function, NIAMDD Nephrology, Urology Research Needs Survey. Vol. 3, pp 69-83 and 100-103.

Burg, M. B., Stephenson, J. L. Transport characteristics of the loop of Henle, Chapter 37, part 3, *Physiological Basis for Disorders of Biological Membranes*, Edited by T. E. Andreoli, J. F. Hoffman, D. Fanestil, Plenum Press. pp 661-679.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03202-08 STB

PERIOD COVERED

October 1, 1978 through September 30, 1979

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	

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:
2.8	2.0	.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 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.

889

PHS-6040
(Rev. 10-76)

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: 1. Improved numerical techniques for the solution of a large system of differential equations have been investigated. Specifically, the analytic expression of the inverse of the pointwise Jacobian matrix has been extended to include a system of m = 6 equations in as many unknowns, namely four solutes, hydraulic pressure and volume flow. The amount of calculation has thus been reduced from $0(m^3/3 + m^2)$ to $0(m^2)$. This scheme has been incorporated into the multinephron full kidney model, a six-tube vasa recta model, and a central core model.

2. For the n interstitial and boundary equations ϕ_G, a numerical Jacobian, Γ, must still be calculated. A rank one update of the inverse Jacobian matrix has been added as follows: Given the *pth* approximation to the interstitial and boundary values, γ_G^p,

$$\gamma_G^{p+1} = \gamma_G^p - H_p \Gamma_p^{-1} \phi_G(\gamma_G^p)$$

where

$$\Gamma_p = (\phi_G(\gamma_G^p + \Delta\gamma) - \phi_G(\gamma_G^p))$$

$H_p = \Delta\gamma I$ for Newton's method

$$= (\gamma_G^p - \gamma_G^{p-1}, \ldots, \gamma_G^{p-\ell} - \gamma_G^{p-\ell+1}, \Delta\gamma e_1, \ldots, \Delta\gamma e_{n-\ell}),$$

$\ell = 1, 2, \ldots, n$ for a modified secant method.

Let

$$\Gamma_p \equiv (q^{p-1}, \ldots, q^{p-n});$$

we may then write

$$\Gamma_{p+1} = \Gamma_p P + (q^p - q^{p-n}) e_1^T.$$

A rank one update of Γ_p^{-1} yields

$$\Gamma_{p+1}^{-1} = P^{-1}\Gamma_p^{-1} - \frac{1}{\alpha} P^{-1}\Gamma_p^{-1}(q^p - q^{p-n}) e_1^T P^{-1}\Gamma_p^{-1}$$

where $\alpha = 1 + e_1^T P^{-1}\Gamma_p^{-1}(q^p - q^{p-n}) \neq 0$.

Updating of Γ_p^{-1} to obtain Γ_{p+1}^{-1} reduces the work required to obtain γ_G^{p+2} from $0(n^3/3 + n^2 + m^2)$ to $0(n^2)$.

This scheme has been incorporated into the full kidney multinephron model, a multinephron central core model and a single nephron vasa recta model.

3. Selected subroutines for matrix manipulation, which are available in LINPACK, have been added to the models. They provide for calculation of condition numbers, which give a measure of the error incurred in solving linear systems; permit in-place LU decomposition of a matrix, essentially halving the storage required to solve such a system; and allow backsubstitution with many right-hand sides, so that as long as the solution of the nonlinear system is being improved, iteration will proceed without recalculation of the Jacobian matrix.

4. Collaborative work has continued with Prof. R. P. Tewarson (SUNY) in the development of computational algorithms having high accuracy, fast convergence, and low storage requirements. During the past year work has centered on methods utilizing cubic and quintic splines. In one method splines are used to compute the numerical derivatives, which are then used to develop formulas having $0(h^6)$ and $0(h^7)$ accuracy. Furthermore, this increase in accuracy can be achieved without any change in the trapezoidal rule Jacobian. The necessary changes are made only to the right hand side (residual vector), before solving the linear system of equations in each Newton step. Of course, this leads to a decrease in the rate of convergence, but possibly this may be offset by the increase in accuracy from $0(h^3)$ - for the trapezoidal rule - to $0(h^6)$ or $0(h^7)$.

Alternatively quintic splines with deficiency can also be used on the y and f values to get comparable accuracy to the cubic spline on spline methods described, where the differential equations are $\frac{dy}{dx} = f$, with $y, f \in R^n$. Furthermore, a seventh degree spline, with deficiency can be used to get higher order schemes with only a small extra computational effort over that required for a quintic spline.

In addition to the work on splines, earlier work has been extended to give a unified derivation of a number of symmetric quasi-Newton update formulas.

5. Data published previously have been verified with the improved models. Comparison of iterative $0(h^3)$-accurate techniques with published variable order results using the six-tube vasa recta model have shown that difficult problems can be solved accurately. For example, a discontinuity in the sources at the junction of the inner and outer medulla can be handled very accurately.

6. A model with direct solute and water exchange in the outer medulla between the vasculature, the ascending limb of Henle and the interstitial space was implemented. Preliminary experiments have shown properties not unlike those of models limited to exchange through the interstitium.

7. The multinephron central core model has been used for additional study of the role of urea cycling in the concentration of urine. These simulations have supported the idea derived from animal experiments and intuitive considerations that the mechanism of urea transport out of the collecting duct is a critical factor.

Proposed Course:

The multinephron model is being modified to take greater account of the detailed architecture of the medullary counterflow system, including a more realistic model of proximal tubule transport. Various improvements of numerical techniques for solving the equations are being developed, and when successful will be incorporated into the models. Additional studies on the problem of microscopic permeabilities to macroscopic renal function are planned.

Publications:

Mejia, R., and Stephenson, J. L. Numerical solution of multinephron kidney equations. J. Comput. Phys. (In press).

Mejia, R., and Stephenson, J. L. Symbolics and numerics of a multinephron kidney model. Proceedings 1979, MACSYMA User's Conference, Washington, D.C. pp 596-603.

*Kellogg, R. B., Tsan, A. Analysis of some difference approximations for a singular perturbation problem without turning points. Math. Comp. 32:1025-1039, 1978.

†Tewarson, R. P. and Farahzad, P. On the numerical solution of differential equations of renal counterflow systems. Computers and Biomedical Research 11:381-391, 1978.

†Tewarson, R. P. A unified derivation of quasi-Newton methods for solving non-sparse and sparse non-linear equations. Computing 21:113-125, 1979.

†Farahzad, P. Analysis for the equations on renal network flows. Math Biosciences 40:233-241, 1978.

†Farahzad, P., and Tewarson, R. P. Numerical continuation method for the solution of a set of parallel flow tubes. Comp. Bio. Med. 9:21-27, 1979.

†Tewarson, R. P. On the use of splines for the numerical solution of non-linear multipoint boundary value problems. College of Engineering, SUNY at Stony Brook, Report No. 329, May 15, 1979.

†Salane, D., and Tewarson, R.P. A unified derivation of quasi-Newton update formulas. J. Inst. Math. Appl. (forthcoming).

Stephenson, J. L., and Mejia, R. Salt water and urea movement in a multi-nephron model of the mammalian kidney. Proc. Internatl. Symposium on Math Topics in Biology, Kyoto, Japan. pp 78-87.

†Supported in part by NHLBI under Contract 201HL03202.
*Supported by NHLBI under Contract HI 52900.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03203-04 STB

PERIOD COVERED

October 1, 1978 through September 30, 1979

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 Associate

COOPERATING UNITS (if any)

Laboratory of Kidney and Electrolyte Metabolism, NHLBI

LAB/BRANCH

SECTION

Section on Theoretical Biophysics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.3	0.2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

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.

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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 the theoretical analysis of transport.

Major Findings: (1) A transporting epithelium has been represented as comprised of a cell and a paracellular channel, both of variable dimensions and bounded by well-stirred mucosal and serosal baths. The variables of the model include concentrations of electrolytes and non-electrolytes, hydrostatic pressure, and electrical potential. Previous work has demonstrated sufficient accuracy when these variables are assumed to be uniform throughout both cell and channel. Boundary conditions are given by the Kedem-Katchalsky relations for membrane transport. For the purpose of simulating certain osmotic experiments, non-electrolyte numerical models of the paracellular channel have been adequate. In this case, transport of a single neutral salt, NaCl, is assumed and electrical effects are not considered.

(2) Development of an analytical non-electrolyte model of the paracellular channel has continued. In this case, a linearized system of membrane transport equations has been used to approximate the Kedem-Katchalsky relations. Previously this linear model has been used to estimate (a) the osmolality (C_R) of the transported salt solution when serosal and mucosal media are equal, and (b) the osmolality difference between mucosal and serosal bathing media (C_M^*) for which the transported solution is identical to the serosal solution. It has been indicated that C_M^* is a more natural measure of epithelial hydraulic leakiness. These results were presented at the International Biophysics Congress in Kyoto (9/78) and in abstract form to the American Society of Nephrology (11/78).

Further applications of the linearized channel model have been to determine the effect of intraepithelial solute polarization on whole epithelial water permeability, to assess the capability of the epithelium to transport water against an osmotic gradient, and to estimate the time course of volume transport out of a finite mucosal medium. It is found that intraepithelial polarization effects may be substantial and that this may serve to rationalize the discrepancy between the high cell membrane water permeabilities necessary to assure isotonic transport (small C_M^*) and the relatively low epithelial water permeabilities that have been reported in the experimental literature. In transport against a gradient, the parameter set used in isotonic transport experiments may yield unsatisfactory predictions for epithelial transport

strength; a variable parameter set, such as is obtained in a model of a compliant channel, yields a more satisfactory result. Estimates of the time course of water transport out of a finite mucosal volume suggest that this may be a useful feature to distinguish flows driven by mucosal hypotonicity from those created by intraepithelial solute-solvent coupling.

The linear non-electrolyte compliant channel model has been analyzed in detail for the description of channel volume transients. Two time constants for the change in channel volume have been identified: a slow process reflecting basement membrane permeabilities and a rapid process whose rate may give a measure of cell membrane water permeability. Experiments to determine these rate constants have been simulated using the full electrolyte epithelial model. Agreement of the numerical simulation with the prediction of the linear analysis suggests that neither electrical effects nor the presence of the cell should greatly confound the experimental observations.

(3) A collaborative project with Drs. Schwartz and Burg of LKEM,NHLBI has involved the simulation of passive transport of CO_2 across proximal tubule in an isolated perfused tubule preparation. The effect of multiple intratubular buffers on the steady-state CO_2 concentration profile has been estimated analytically. This analysis has been incorporated into a numerical model that determines the tubule permeability to CO_2 as a function of the perfusion conditions and the observed collected fluid pH; the model is used in the reduction of experimental data. It was observed that for the experiments considered, the standard assumption of an exponential CO_2 concentration profile along the tubule length, gave permeability errors no greater than 6%.

The CO_2 flow within the collecting pipets used by Drs. Schwartz and Burg has been simulated numerically to estimate the error in pH determinations due to CO_2 loss into the oil meniscus. It is found that for perfusion rates greater than 1 nl/min, this error is less than 2%.

Proposed Course:

Collaborative work is planned with Dr. Spring of LKEM,NHLBI for measurements of channel volume transients in order to estimate cell membrane water permeability. Work will continue on the use of linear approximate models as an aid to understanding the behavior of more complex non-linear epithelial phenomena.

Serial No. Z01 HL 03203-04 STB

Publications:

Huss, R. E., and Stephenson, J. L. A mathematical model of proximal tubule absorption. J. Membrane Biology 47:377-399, 1979.

Weinstein, A. M., and Stephenson, J. L. Electrolyte transport across a single epithelium: Steady state and transient analysis. Biophysical Journal (in press).

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, 1978 to September 30, 1979

The Section functions primarily in a support role to all laboratories of IR providing care for many species of animals, technical assistance in preparation and maintenance of animal models for various experimental regimens, and the development of animal resources not otherwise available.

Maintenance of various rodent and aquatic 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, and the NIHAC. Postoperative intensive care and treatment of surgery patients is completed in Buildings 3 and 28.

The animal surgery laboratory located in Building 14-E supports the Clinical Hematology Branch, Laboratory of Experimental Atherosclerosis, Laboratory of Technical Development, the Surgery Branch, and the Pulmonary Branch in preparation of experimental animal models, completing cardiovascular studies requiring animal models 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.

The NHLBI Sheep Colony has continued successful 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.

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, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-03401-03-LAMS

PERIOD COVERED
10/1/78 through 9/30/79

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
Other:	D. K. Buckhold	SLAMS, NHLBI
	W. C. Roberts	Chief, LP, NHLBI
	V. J. Ferrans	LP, NHLBI
	J. H. Bell	SB, NHLBI
	M. A. Borkon	SB, NHLBI
	M. Jones	SB, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Office of the Director of Intramural Research

SECTION
Section on Laboratory Animal Medicine and Surgery

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

Cardiac catheterization of more than 65 dogs has demonstrated varying peak systolic pressure gradients between left ventricle and aorta from < 5 to > 150 mmHg. Laboratory studies are presently underway to define hemodynamic parameters and tissue morphology resultant of Newfoundland discrete 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.

901

PHS-6040
(Rev. 10-76)

Project Description: Z01-HL-03401-03-LAMS

The Newfoundland Breeding Colony was maintained by Flow Laboratories, Inc., at Dublin, Virginia supported by NIH 263-78-D-0253. Twenty two dogs remain and are located in Bldg. 28.

Varying degrees of severity of LVH existed in dogs studied from the colony and gross and microscopic changes of heart tissue existed similar to those occurring in human patients with similar cardiac abnormalities.

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.

More than 65 cardiac catheterizations have demonstrated peak systolic blood pressure gradients between left ventricle and aorta (LV-aorta PG) range from < 5 mmHg to > 150 mmHg.

All hearts grossly examined have demonstrated a fibrous membrane located 3-8 mm below the sinus of valsalva. The discrete narrowing varies in different hearts. Severely effected LV outflow tracts have circumferential discrete narrowing and extend transversely from the anterior mitral leaflets at the level of its attachment to the atrial septum and the outflow surface of the leaflet. Some lesions in the more severely affected dogs appear to be similar to a condition described in human heart disease known as tunnel subaortic stenosis.

Varying degrees of concentric left ventricular hypertrophy occurs: (1) in all dogs > 3 years old; (2) in all age groups with LV-aorta PG > 80 mmHg; (3) Severe hypertrophy is present in dogs several years old with LV-aorta PG > 125 mmHg.

Histologic and ultrastructural studies showed that the subaortic fibrous "ring" present in Newfoundland dogs with discrete SAS lacked the layered structure of normal endocardium in left ventricular outflow tract. The fibrous ring tissue was characterized by the presence of large uni- and multinucleated, rounded connective tissue cells that resembled chondrocytes in several respects. The cells were surrounded by connective tissue rich in acid mucophysaccharides, small but cross-banded collagen fibrils and small, poorly developed elastic fibers. The unusual differentiation of cellular and extracellular components of connective tissue in subaortic fibrous rings in dogs with discrete SAS clearly differs from that seen in humans with discrete SAS.

Morphologic studies of small intramural coronary arteries revealed a > 50% incidence of narrowing of these vessels by proliferation of smooth muscle cells (SMC) in intima and by fibrosis involving intima, media, and adventitia. The

most striking ultrastructural abnormality involves intimal elastic tissue which forms numerous small, scattered fibrils (> 1μ in diameter) rather than discrete laminae separating layers of SMC. Collagen fibers between SMC are increased in number but are morphologically normal. The changes described resemble those in humans with hypertrophic cardiomyopathy and with discrete ring or tunnel types of SAS.

Dogs with pressure gradients above 150 mmHg are susceptible to syncope, "Sudden Death Syndrome" or atrial fibrillation and eventual death from heart failure.

Breeding has been terminated until laboratory studies are completed and evaluated. Future breeding will be considered if the "defined" animal model is determined useful for study of the hypertrophied ventricle secondary to subcoronary LV outflow tract obstruction.

Contract Information:

Contract Number:	263-78-D-0253	
Period Covered:	7/1/78 through 8/31/79	
Newfoundland Colony:	< $51,000	
Contractor:	Flow Laboratories, Inc. Dublin, Virginia	
PI:	W. Knapp	
Manyears (est'd):	Professional:	0.1
	Other:	2.0
	Total:	2.1

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-03402-03-LAMS

PERIOD COVERED
10/1/78 through 9/30/79

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, OD, NHLBI

Other: J. F. Harwell, Jr. SLAMS, OD, NHLBI

COOPERATING UNITS (if any) (1) Laboratory of Biomedical Sciences, IRP, NICHHD, (2) NINCDS, IRP, LPP, (3) Uniformed Services University of the Health Sciences (4) DOD - USAMRIID, Animal Assessment Div., Ft. Detrick, MD, (5) DOD - National Naval Medical Center, USN

LAB/BRANCH
Office of Director of Intramural Research

SECTION
Section on Laboratory Animal Medicine and Surgery

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

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; the Laboratory of Biomedical Sciences, IRP, NICHHD; LPP, IRP, NINCDS. 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 existance of varied states of health in animals delivered for laboratory use.

904

PHS-6040
(Rev. 10-76)

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 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 ovine and bovine bacterins, and tetanus toxoid 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 weekly 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 exams 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 states. Microbiological and serological screening for detection of suspected disease entities is carried out when indicated.

The colony represents the "sol" source of laboratory sheep in the Washington area available 12 months a year to meet various requirements of NIH research programs supplied. Husbandry techniques are in effect 365 days per year to meet the everchanging requirements of research programs.

This project will continue as long as IR and other NIH programs have sufficient requirements that justify the continued support of this laboratory sheep resource and it does not become cost prohibitive. Production goals and total numbers of animals maintained will be varied as required by changing demands of laboratories.

Contract Information: Z01-HL-03402-03-LAMS

Contract Number: N01-HI-7-2900 - approximately $175,000 - 1978 through 1979

Contract Site: Double J Farms
Luray, Virginia

PI: Jesse Judy, Contractor
Donna Matthews, Colony Manager

Total Manyears: 7.0

Professional: 2.0

Other: 5.0

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-03403-02-LAMS

PERIOD COVERED
March 1979 through September 1979

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

J. E. Pierce	Chief, SLAMS, OD, NHLBI
D. K. Buckhold	SLAMS, OD, NHLBI
J. F. Harwell, Jr.	SLAMS, OD, NHLBI

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
National Heart, Lung, and Blood Institute, 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. The level where maximum oscillations are observed is designated mean arterial 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 designed 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.

907

Project Description: Z01-HL-03403-02-LAMS

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.

A commercially available unit using the principle of oscillometry is being used and although satisfactory results have been obtained in both awake and anesthetized dogs, modification of the technique is required. Following positioning of the cuff, the unit performs independently: (1) inflates the cuff well above normal systolic pressure, (2) gradually releases cuff pressure at increments of 3-7 mmHg, (3) a pressure transducer monitors cuff pressure and detects oscillations in the ranges they occur related to systemic arterial blood pressure, (4) a microprocessor detects changes in oscillations and reports systolic, diastolic and mean blood pressure values by digital readout, (5) automatically recycles as required (from 30 seconds to several minutes) presenting updated pressure measurements.

Use of this technique will continue and instrumentation will be modified in an attempt to overcome recognized problems that exist when obtaining pressure measurements on animals

Annual Report of the
Pulmonary Branch
National Heart, Lung, and Blood Institute
October 1, 1978 through September 30, 1979

The major function of the lung is to exchange gases between the atmosphere and blood. To accomplish this, the lung has evolved as a complex structure which brings together the atmosphere and the blood in a fashion which is closely regulated to insure maximum efficiency. The major thesis of this laboratory is that the process of gas exchange is critically dependent on the ability of lung cells to maintain lung structure and function and on the inflammatory and immune systems to protect the lung against a variety of insults. The adult lung consists of approximately 40 cell types, a complex extracellular matrix, and a sophisticated inflammatory and immune system that can vary independently of its systemic counterpart. Of particular importance to lung structure and function is the ability of the lung to maintain its connective tissue, as these components provide a scaffolding to define lung structure and are critical determinants of the mechanical properties of lung during the processes of gas exchange. Disturbances of the connective tissue of the lung, such as that found in emphysema or the interstitial lung disorders, has profound effects on the ability of the lung to function as an efficient organ of gas exchange.

As a comprehensive approach toward understanding the composition and regulation of lung structure in health and disease, the Pulmonary Branch utilizes several approaches, including:

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, 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) fibronectin, a newly described class of glycoproteins involved in cell-cell and cell-matrix interactions. In the past year, our studies have concentrated on the control of connective tissue production and destruction. The major findings in this area have been as follows:

(1) Modulation of collagen production in cultured human lung fibroblasts. Methods have been developed to culture fibroblasts from human lung tissue of normal individuals as well as those with a variety of lung disorders. These fibroblasts have been extensively characterized and several are now maintained in the permanent collection of the American Type Culture Collection. These fibroblasts have been evaluated in terms of their ultrastructure as well as a variety of characteristics of their differentiated state. All the human lung fibroblasts studied to date produce both collagens I and III and no differences in the types of collagen produced have been detected in fibroblast cultures from patients with various interstitial lung disorders.

(2) Evaluation of collagen production in human fetal lung of fibroblasts has demonstrated that they produce the same quantity of collagen throughout their growth curve and as well as over at least 25 population doublings. This invariance of collagen production suggests the production of this macromolecule is tightly controlled as befits its critical importance in maintaining lung structure and function.

(3) Evaluation of cyclic AMP levels in human lung fibroblasts has demonstrated that this cyclic nucleotide is critically linked to collagen production. Agents that increase intracellular cyclic AMP (e.g., isoproternol, PEG_1, cholera toxin) all decrease collagen production by these fibroblasts. Other β-stimulents such as norepinephrine and epinephrine also caused this phenomena. The concept has been developed that under normal conditions fibroblasts of the body are generally "suppressed" in terms of their potential for collagen production by endogenous β-stimulants. This suggests that the β-adrenergic system "holds back" collagen production and thus has the potential for significantly modifying the quantity of extracellular matrix components. This also leads to possible mechanism of β-agonist-induced fibrosis, i.e., when propanolol is given to a susceptible individual, this β-blocker prevents the normal suppression of collagen production by endogenous β-agonists and thus increases the production of collagen in certain organs.

(4) It is clear now that a significant proportion of collagen produced by fibroblasts are degraded within the cell prior to secretion. This appears to be a potent control mechanism for modulating the amounts of intact collagen that are produced by the cells. Studies with proline analogs (e.g., azedetine, cis-hydroxyproline) cause collagen to be defective resulting in a marked increase in intracellular degradation. "Defective" collagen can also be produced in lung fibroblasts by omitting ascorbic acid, a cofactor for the normal hydroxylation of proline residues, a post-translational mechanism that is critical for normal collagen helical structure. Without ascorbic acid these cells produce proportionally increased amounts of defective collagen and intracellular collagen degradation increases 2 to 3 fold. Studies have also shown that at least part of the modulation of collagen production by fibroblasts by cyclic AMP is modulated through this intracellular degradative process. Intracellular collagen degradation appears to occur, at least in part, in lysozomes, as studies with lysozomal inhibitors indicate that the intracellular degradation of defective collagen can be markedly inhibited. Additional studies of fibroblasts from individuals with osteogenesis imperfecta have shown that intracellular collagen degradation is the same in these cells as in those from normal individuals.

(5) Studies of collagen production in tendon, skin, and lung of sheep of varying gestational age have demonstrated that messenger RNA levels significantly control the production of collagen by these tissues. The percentage of protein synthesis devoted to collagen production varies in these tissues as a function of gestational age during fetal development, and appears to be paralleled in general by collagen messenger RNA levels. The exception is in skin, where high messenger RNA levels are maintained late in gestational life whereas the tissue appears not to be using all the messenger RNA that is present.

(6) Studies are in progress concerning the Ehlers-Danlos IV syndrome, an hereditary disorder of connective tissue in which type III collagen is not made. Messenger RNA for collagen can be isolated from fibroblasts from these patients and are being compared to normal fibroblasts. To search for the underlying genetic defect in this disorder, studies are utilizing these fibroblasts to isolate messenger RNA for collagen types I and III and to evaluate type I and type III collagen gene structure.

(7) Studies are ongoing to evaluate the production of elastin by a variety of tissue including lung explants and smooth muscle cultures. It is clear that elastin is produced as a 70,000 dalton product. Peptide maps have been developed to evaluate the structure of this protein and compare it from tissue to tissue. In addition, an elastin messenger RNA has been prepared as has an elastin complementary DNA. These components are currently being used to evaluate elastin gene structure and the control of elastin biosynthesis.

(8) Detailed studies are ongoing to evaluate the control of collagen production at the gene level. Using the techniques of recombinant DNA, approximately 50% of the sheep pro $\alpha 2$ gene has been isolated. Whereas the coding sequences for the 3' 50% of the pro $\alpha 2$ gene is approximately 2,500 bases, restriction map studies have shown that this structural information is spread out over at least 14,000 bases. Mapping of this genomic DNA using electron microscopic techniques has demonstrated that there are at least 12 intervening, non-coding sequences spread out through this 14,000 base region. Detailed restriction maps have been prepared and are being used to evaluate the fine structure of this gene together with DNA sequencing of selected regions. Studies are continuing to isolate the genes for other major connective tissue components found in lung and these are being evaluated using similar technology.

(9) Studies of connective tissue destruction have concentrated primarily on evaluating the sensitivity of fibronectin to proteases released from the peripheral blood neutrophil. Fibronectin, a critical connective tissue component that appears to be important in cell-cell and cell-matrix interactions, is exquisitely sensitive to the neutral proteseases of the neutrophil suggesting a mechanism by which neutrophils in the lung may significantly derange lung structure by attacking an important connective tissue matrix component.

(10) Studies of collagenase in the human peripheral blood neutrophil have shown that it is present in both the primary and secondary granules. These sites appear to harbor different collagenases as the collagenase in the primary granule attacks both collagen types I ad III whereas the collagenase in the secondary granule attacks type I only. In addition, these collagenases appear to have different inhibitory spectra, molecule weights and relative states of activation.

II. Relationship of the Inflammatory and Immune Systems to the Maintenance of Lung Structure and Function

The inflammatory and immune systems of lung are important determinants of lung structure and function in health and disease. The Pulmonary Branch has instituted a broad base 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 complements 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. Using these techniques, major insights have been made concerning the inflammatory and immune systems of the lung parenchyma in both health and disease (see also Project No. Z01 HL 02405-06 PB). In the past year, these studies have included:

(1) In the normal human lung there are basically two effector cells present: the alveolar macrophage and the lymphocyte. The macrophage represents approximately ninety percent of the inflammatory and immune effector cells in the lower respiratory tract. It is originally derived from the circulating blood monocyte. Studies of a patient receiving a bone marrow transplantation from a sibling of the opposite sex has demonstrated that the human alveolar macrophage can persist in the lower respiratory tract for a period of at least four years. These macrophages have receptors for the Fc portion of IgG and for the C3b component complement. Macrophages can perform as potent effector cells in antibody dependent cellular cytotoxicity. These macrophages can also be induced to provide a variety of enzymes that are important in the inflammatory process. A great deal of effort has gone into evaluating the role of the alveolar macrophage in attracting other cells to the lower respiratory tract. Most importantly, both animal and human alveolar macrophages produce a chemotactic factor that is relatively specific for the circulating blood neutrophil. This chemotactic factor is of small molecular weight (600-800 daltons) and is at least partially lipid in nature. The macrophage can be stimulated to produce this chemotactic factor by a variety of agents including microorganisms, noninfectious particulates, and immune complexes of the IgG type. (See also Project No. Z01 HL02405-06 PB).

In comparison to the macrophage, the lymphocyte comprises approximately ten percent of the total effector cells in the lower respiratory tract. Of these lymphocytes, approximately seventy percent are T-lymphocytes, and eight percent are B-lymphocytes. Of the T-lymphocytes, approximately five percent appear "activated" (i.e., have Fc receptors for IgG and will rosette with sheep red blood cells at 37^{o}). A reverse hemolytic plaque assay has been developed to quantitate the number and types of immunoglobulins produced by lung B-lymphocytes. Approximately 0.5 percent of the B-lymphocytes in the lower respiratory tract are actively producing immunoglobulin. Comparison with blood has shown that these levels are comparable and include all the major immunoglobulin classes.

Studies of cell interactions among the effector cells comprising the lower respiratory tract have shown that the macrophage not only recruits the neutrophil to the lower respiratory tract via a chemotactic factor but this chemotactic factor also "activates" the neutrophil to release its array of a variety of mediators (including its preformed enzymes such as collagenase and elastase, both of which are important in terms of the maintenance of connective tissue structure and function in lung). Studies of macrophage-lymphocyte interactions have shown that, as in other systems, the macrophage is an important "accessory" cell for the responsiveness of the lymphocyte to mitogens and antigens.

(2) Two animal models have been used to evaluate inflammatory and immune processes in the lower respiratory tract. The first is bleomycin, an antibiotic which is used as an antitumor agent and causes interstitial lung disease in both humans and animals. Studies of normal versus nude, athymic mice (these mice lack a T-cell system and hence lacks cellular-immune processes) has shown that these animals are as susceptible to bleomycin as are their normal counterparts. This suggests that although the cellular immune system may be important in terms of the development of the bleomycin lesion, it is not a necessary component and that alternate mechanisms can take over. A model of acute hypersensitivity pneumonitis has been developed using animals immunized against ovalbumin and given intratracheal particulate ovalbumin. This model has been used to quantitate the types and function of inflammatory and immune effector cells present in the lung at different times after the lesion is induced. Most strikingly, there is an initial neutrophil inflammation which soon converts to a mononuclear cell inflammatory process. Evaluation of these mononuclear cells has shown that there is a rapid rise in the proportion of T-lymphocytes which then goes back to normal by two weeks after the lesion begins. Evaluation of the macrophages soon after addition of the intratracheal ovalbumin has shown that these macrophages are producing a chemotactic factor for neutrophils and hence are likely responsible, at least in part, for the traffic of these inflammatory cells from blood to lung in the early stages of this lesion.

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.

The interstitial lung disorders represent 15-30% of the noninfectious disorders of lung. Thre 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 inhalants, 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 a collagen-vascular disorder and sarcoidosis. In the past year, the Pulmonary Branch has continued its detailed studies of patients with interstitial disease with particular emphasis on those with IPF and sarcoidosis. The highlights of these studies include:

(1) Detailed physiologic studies of patients with IPF have shown that it is very difficult to estimate the extent of inflammation and fibrosis in the lung using physiologic studies. The conventional physiologic parameter such as vital capacity, total lung capacity, diffusing capacity, and resting arterial blood gases have no relationship to the extent of disease as estimated by open lung biopsy. However, static deflation volume-pressure relationships do correlate with the degree of fibrosis as does the extent of drop of arterial oxygen tension with exercise. However, the latter is not specific for the fibrosis as it also correlates well with the extent of inflammation. In

studies in collaboration with the Clinical Hematology Branch, NHLBI, the Pulmonary Branch has been evaluating conventional steady state exercise methodology with a newer breath by breath exercise protocol using an increasing amounts of work load throughout the test. The latter has the advantage of being more rapid and in certain studies, noninvasive. In conjunction with the Critical Care Medicine Group, Clinical Center, the Pulmonary Branch has been evaluating the pulmonary circulation of patients with interstitial lung disease. Quite remarkably, their pulmonary hemodynamics are quite similar to patients with panacinar emphasema and different from those with chronic obstructive lung disease of the bronchitic variety. In the past year, methods have been developed to evaluate the work of breathing and to use this as a parameter of the extent of disease in these individuals. While preliminary studies suggest it is not very sensitive to the development of disease, it may prove useful for following response to drug therapy.

(2) Inflammatory and immune processes. As described in section II above, a major development has been the adaptation of the fiberoptic bronchoscope to evaluate the inflammatory and immune processes of the lower respiratory tract in humans. This technique, however, relies on the assumption that the cells and noncellular components that are washed off the epithelial surface of the lower respiratory tract reflect those inflammatory and immune processes that are ongoing throughout the lung parenchyma. To test this hypothesis, methods have been developed to directly isolate inflammatory and immune effector cells directly from the lung of patients undergoing open lung biopsy for diagnostic purposes. These comparisons have shown that the lavage method gives an accurate reflection of the inflammatory and immune effector cells present and thus is a valid means to assessing individuals with these disorders. One of the most important accomplishments of the past year has been the characterization of the chronic inflammation in the lower respiratory tract of patients with interstitial lung disease using lavage and biopsy methods. Compared to normal individuals (see II above) patients with interstitial disease have increased numbers of inflammatory and immune effector cells in their lower respiratory tract. In addition, there are significant changes in the proportions of these cells present and such changes are likely the key to the pathogenesis of these diseases. Basically, the interstitial disorders that have been evaluated can be grouped into two categories of inflammation: a neutrophil type of inflammation and a lymphocyte type of inflammation. In the first, as typified by IPF, there is a chronic accumulation of neutrophils in the lower respiratory tract. Whereas normally less than 1% of the inflammatory and immune effector cells present are neutrophils, in diseases like IPF, 5 to 25% of the effector cells are neutrophils. In contrast, in patients with sarcoidosis and hypersensitivity pneumonitis, there are no neutrophils but very high proportions of lymphocytes. Whereas normally, lymphocytes comprise less than 10% of the total inflammatory and immune effector cells, in these disorders lymphocytes comprise greater than 15% (often up to 50 to 60%) of the total cells present. Not only are there increased numbers of lymphocytes, but the relative proportions of types of lymphocytes change such that there are more T-lymphocytes. These T-lymphocytes often appear activated, i.e., increased numbers and receptors for the Fc portion of IgG and rosette with sheep red blood cells at 37°. As more interstitial disorders are evaluated, it is likely we will be able to characterize them on the basis of the inflammatory and immune effector cell

subtypes that are present. These methods have also given insights into how the chronic inflammation is maintained. In idiopathic pulmonary fibrosis, studies of macrophages have shown that they have immune complexes on their surface and that these immune complexes induce the macrophages to release a chemotactic factor specific for neutrophils. This likely explains the mechanism by which neutrophils are chronically attracted to the lung in this disorder. In addition, while some of these patients have circulating immune complexes, a large proportion have immune complexes within the lower respiratory tract, suggesting that the immune complexes are being formed within the lung and not brought to the lung from elsewhere in the body. In addition, the use of a reverse hemolytic plaque assay has demonstrated that large numbers of B-lymphocytes from the lungs of patients with idiopathic pulmonary fibrosis are making immunoglobulin compared to the B-lymphocytes of peripheral blood. Although the largest proportion of B-lymphocytes are producing IgG, significant numbers are also producing IgM or IgA. Whereas in peripheral blood, approximately $500/10^6$ lymphocytes are producing IgG, up to $30,000/10^6$ lymphocytes are producing IgG within the lungs of patients with IPF. Additional studies have demonstrated that the macrophages in these patients interact with neutrophils to induce the neutrophil to destroy lung fibroblasts. In contrast to idiopathic pulmonary fibrosis, the maintenance of the inflammation in sarcoidosis is likely mediated by the activated T-lymphocyte. These cells are producing a chemotactic factor for blood monocytes. This is of critical importance in a disease like sarcoidosis, as the attraction and activation of blood monocytes is important for the formation of granulomata which are characteristic of this disease.

(3) Morphology. In conjunction with the Pathology Branch, NHLBI, detailed studies have been ongoing as to the ultrastructure of the lung in health and disease. A new connective tissue structure, termed "anchoring fibrils" has been defined in the alveolar interstitium of patients with interstitial disease. These 200-600 angstrom wide, 4000-6000 angstrom long structures seem to insert into the basement membrane under sites of cuboidal epithelial cells in the regions of hemi-desmosomes. Similar structures are found in the tracheabronchial tree of the normal dog. In the latter circumstance, however, they are decreasing in numbers as one progresses down the tracheabronchial tree and are not found in the normal alveolar structures. Additional studies have shown that patients with interstitial lung disease have surprising nuclear inclusions which appear to be infoldings of the nuclear membrane. The reason for these are not known but are quite characteristic and found in up to 1% of the nuclei of the epithelial cells of the alveolar structures.

(4) Therapy. Current therapeutic protocols for the treatment of IPF include: (a) cyclophosphamide versus prednisone; (b) intramittent intravenous large bolus corticosteroids; and (c) dapsone, a sulfone used therapeutically to treat other disorders in which there seems to be an accumulation of neutrophils associated with immune complexes (as in the case of the lung in IPF). In sarcoidosis, current therapeutic protocols include: (a) the double blind use of intermittent high dose intravenous corticosteroids for a short period; and (b) the prospective evaluation of the prognostic significance of high intensity inflammation in the lung compared to low intensity inflammation in the lung with or without corticosteroid intervention.

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 trafficking to, the lung balanced by anti-proteases (i.e., α1-antiproteinase, a circulating antiprotease that diffused 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.

(1) Smoking. Evaluation of alveolar macrophages recovered from smokers and nonsmokers have shown that the macrophages of smokers are spontaneously releasing a chemotactic factor for neutrophils. This likely explains why 2-3% of the inflammatory and immune effector cells of the lower respiratory tract of smokers are neutrophils, whereas less than 1% are so in non-smokers. In vitro studies of alveolar macrophages obtained from normal individuals have shown that, when exposed to cigarette smoke in culture dishes, normal alveolar macrophages will also produce this neutrophil chemotactic factor. It has been hypothesized, that in addition to having more neutrophils in their respiratory tract, that smokers are more at risk for lung destruction because their anti-proteases are not as effective as those of nonsmokers. To evaluate this hypothesis, the anti-elastase activity of components of lavage fluid of smokers was compared to nonsmokers for the effectiveness of the α1-antiproteinase within the lower respiratory tract. Quite strikingly the α1-antiproteinase within the lavage fluid of the smokers was 50% as effective as an anti-elastase as was the material from nonsmokers. Thus it appears that the lungs of smokers have less protection against proteolytic attack than do the lungs of nonsmokers.

(2) Therapy. The heredity disorder α1-antitrypsin deficiency is a disease in which those affected have a marked reduction of this serum glycoprotein by both functional and antigen criteria. Normally α1-antitrypsin is the principle serum protease inhibitor. In the homozygous deficiency state (phenotype "ZZ"), 80% of affected adults have accelerated, usually fatal, destructive lung disease. Although considerable progress has been made in characterizing this protease inhibitor abnormality, clues to the precise pathophysiology and effective therapy for this disorder are lacking. Current indications suggest that the ZZ homozygous protein is a single amino acid substitution. Most likely, this abnormality results in changes in intra-cellular confirmation of the molecule such that sugar side chains cannot be added. Consequently the altered ZZ protein cannot be secreted in the normal fashion by the liver cells in which it is made. A drug trial with danazol, a "impeded" androgen which increases antiprotease release by the liver, has demonstrated that this drug will increase the levels of α1-antitrypsin in the serum of patients with α1-antitrypsin deficiency by 40%. Whether or not

these increases will be efficacious for the therapy of this disease will require further detailed study. In addition, studies are ongoing to "cure" this disorder by direct replacement of the missing α1-antitrypsin. A protocol has been developed where partially purified α1-antitrypsin is given intravenously on a once a week basis for four weeks to patients homozygous with this disease. The purpose of the protocol is a pilot study to evaluate the feasability of maintaining serum levels over a period of time in these patients. Initial indications suggest that with a once a week administration of the equivalent of the α1-antitrypsin in two liters of plasma, "above threshold" levels can be maintained.

(3) Other studies have identified a new form of destructive lung disease not generally considered to be in this category. Evaluation of patients with acquired hypogammaglobulinemia have demonstrated that approximately 25% of patients with long standing immunoglobulin deficiency have a lower lobe emphysema by chest film. This lung destruction looks indistinguishable from those of patients with α1-antitrypsin deficiency. In patients with acquired hypogammaglobulinemia, however, the lung destruction is likely secondary to repeated bouts of infection (as the primary pathogenic event) initiating an increased protease burden and thus the protease anti-protease imbalance.

There are several forms of lung disease which have a known hereditary basis. By evaluating these "experiments of nature" we can derive an understanding of the structure and function of the lung which can then be applied to more general understanding of pulmonary disorders. In addition to α1-antitrypsin deficiency discussed above, the following disorders have been chosen as models of more generalized forms of lung disease:

(1) Fabry's Disease, Angiokeratoma Corporis Diffusion Universlae, is a sex-linked sphingolipid disorder. The lipid which accumulates is ceramide trihexoside and the deficient enzyme is ceramide trihoxosidase. Studies of Fabry's Disease has shown that there is abnormal lipid deposition in the airway epithelial cells. Correlates with functional abnormalities of the airways have shown that it likely is the basis for obstructive lung disease in these patients. In addition, correlation with smoking history has demonstrated that the patient with Fabry's Disease is greatly at risk if he smokes, since the airway obstruction seen with these patients is far beyond that attributable to smoking alone. Thus, it is likely that smoking thrusts these patients above a lowered threshold for the development of obstructive lung disease and suggests that Fabry's Disease could be added to the list of those genetic disorders associated with obstructive lung disease.

(2) Hereditary disorders of connective tissue. The connective tissue of lung is complex, consisting of four genetically distinct types of collagen, elastic fibers, seven types of glycosaminoglycans with their associated proteins and a variety of other macromolecules composing the ground substance. This complexity, together with the intricate anatomy of lung parenchyma, makes the study of the influence of connective tissue on the control of lung structure and function extraordinarily difficult. The major objectives of this proposal are attempts to use "experiments of nature" involving connective tissue (Marfan Syndrome, Ehlers-Danlos Syndrome, Osteogenesis Imperfecta)

to help understand the role each connective tissue component plays in the maintenance of lung structure and function. The lung is the only organ of the body where it is relatively easy (using the sophisticated methodology of pulmonary function testing) to make direct inference on the role of connective tissue in controlling mechanical properties. Thus, the lung affords an opportunity for understanding the biochemical-mechanical correlates of connective tissue. This project is done in conjunction with biochemical studies on the control of synthesis and destruction of connective tissue (see Project No. Z01 HL 02409-04 PB). Patients with specific connective tissue disorders are admitted to the Pulmonary Branch Clinical Service and pulmonary function tests are used to evaluate the "mechanical" status of their lungs. This is a long-term project but with the methodology available today, it should be possible to specifically define the role of each connective tissue component in lung and demonstrate how these components describe pulmonary abnormalities in these hereditary disorders.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02405-06 PB

PERIOD COVERED

October 1, 1978 to September 30, 1979

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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	I. Krefting	Clinical Associate	NHLBI PB
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COOPERATING UNITS (if any)

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Pulmonary Branch

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
9.3	6.3	3.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

It is clear that the inflammatory and immune systems are critically important for the maintenance of lung structure and function in health and disease. The Pulmonary Branch has instituted a broad based program to evaluate the composition and function of the cells comprising these systems in animal models and in both normal and diseased human lung. Methods have been developed to define these cell populations and evaluate their effector function. Particular attention is being paid to macrophage production of factors chemotactic for neutrophils, influence of immune effector cells on connective tissue production by mesenchymal cells, evaluation of the inflammatory and immune effector cells comprising the alveolitis of idiopathic pulmonary fibrosis and sarcoidosis, and animal models of hypersensitivity pneumonitis.

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(Rev. 10-76)

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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 started 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) develop animal models of interstitial lung disease in which the influence of the inflammatory and immune systems can be evaluated; (2) evaluation of the mechanisms of inflammatory and immune effector cell traffic into the alveolar structures; (3) evaluation of the influence of immune effector cells on extracellular matrix macromolecule production by mesenchymal cells; and (4) evaluation of the immune and inflammatory effector cells comprising the alveolitis of the human interstitial lung disorders (see also Project No. Z01 HL 02407-05 PB).

Methods: The techniques used for these studies are standard in the fields of inflammation and immunology. For animal studies of interstitial lung disease two stimuli were used: (1) bleomycin, an anti-tumor antibiotic known to cause interstitial lung disease in 10% of patients receiving it; (2) particulate ovalbumin in previously sensitized animals; the particulate ovalbumin is used as an analogue of antigens in the hypersensitivity lung disorders. For the bleomycin studies, mice are utilized that have carefully defined genetic strains lacking a component of the immune system. For the hypersensitivity studies, inbred strains of guinea pigs are utilized. To evaluate the mechanism of the inflammatory immune effector cell traffic into the alveolar structures, alveolar macrophages derived from the guinea pig lung were cultured in vitro with various agents used as analogues of stimuli causing human disorders. In addition, alveolar macrophages from normal human volunteers were utilized in vitro in similar studies. In both of these situations, various target cells were used to evaluate the possible chemotactic factors produced by the alveolar macrophages under various states of stimulation. To evaluate the inflammatory immune effector cells comprising the alveolitis of the human interstitial lung disorders (see also Project Z01 HL 02407-05 PB), two methodologies were utilized: (1) bronchoalveolar lavage via the fiberoptic bronchoscope; and (2) open lung biopsy. In both cases inflammatory and immune effector cells were recovered, purified by gradient centrifugation and subtypes of the cells purified by conventional means. These cell populations were then evaluated for types of cells present and effector function of the cells.

Major Findings:

(1) In the normal human lung there are basically two effector cells present: the alveolar macrophage and the lymphocyte. The macrophage represents approximately ninety percent of the inflammatory and immune effector cells in the lower respiratory tract. It is originally derived from the circulating blood monocyte. Studies of a patient receiving a bone marrow transplantation from a sibling of the opposite sex has demonstrated that the human alveolar macrophage can persist in the lower respiratory tract for a period of at least four years. These macrophages have receptors for the Fc portion of IgG and for the C3b component of complement. Macrophages can perform as potent effector cells in antibody dependent cellular cytotoxicity. These macrophages can also be induced to provide a variety of enzymes that are important in the inflammatory process. A great deal of effort has gone into evaluating the role of the alveolar macrophage in attracting other cells to the lower respiratory tract. Most importantly, both animal and human alveolar macrophages produce a chemotactic factor that is relatively specific for the circulating blood neutrophil. This chemotactic factor is of small molecular weight (600-800 daltons) and is at least partially lipid in nature. The macrophage can be stimulated to produce this chemotactic factor by a variety of agents including microorganisms, noninfectious particulates, and immune complexes of the IgG type. (See also Project No. Z01 HL 02407-05 PB).

In comparison to the macrophage, the lymphocyte comprises approximately ten percent of the total effector cells in the lower respiratory tract. Of these lymphocytes, approximately seventy percent are T-lymphocytes, and eight percent are B-lymphocytes. Of the T-lymphocytes, approximately five percent appear "activated" (i.e., have Fc receptors for IgG and will rosette with sheep red blood cells at 37°). A reverse hemolytic plaque assay has been developed to quantitate the number and types of immunoglobulins produced by lung B-lymphocytes. Approximately 0.5 percent of the B-lymphocytes in the lower respiratory tract are actively producing immunoglobulin. Comparison with blood has shown that these levels are comparable and include all the major immunoglobulin classes.

Studies of cell interactions among the effector cells comprising the lower respiratory tract have shown that the macrophage not only recruits the neutrophil to the lower respiratory tract via a chemotactic factor but this chemotactic factor also "activates" the neutrophil to release its array of a variety of mediators (including its preformed enzymes such as collagenase and elastase, both of which are important in terms of the maintenance of connective tissue structure and function in lung). Studies of macrophage-lymphocyte interactions have shown that, as in

other systems, the macrophage is an important "accessory" cell for the responsiveness of the lymphocyte to mitogens and antigens.

(2) Two animal models have been used to evaluate inflammatory and immune processes in the lower respiratory tract. The first is bleomycin, an antibiotic which is used as an antitumor agent and causes interstitial lung disease in both humans and animals. Studies of normal versus nude, athymic mice (these mice lack a T-cell system and hence lacks cellular-immune processes) has shown that these animals are as susceptible to bleomycin as are their counterparts. This suggests that although the cellular immune system may be important in terms of the development of the bleomycin lesion, it is not a necessary component and that alternate mechanisms can take over. A model of acute hypersensitivity pneumonitis has been developed using animals immunized against ovalbumin and given intratracheal particulate ovalbumin. This model has been used to quantitate the types and function of inflammatory and immune effector cells present in the lung at different times after the lesion is induced. Most strikingly, there is an initial neutrophil inflammation which soon converts to a mononuclear cell inflammatory process. Evaluation of these mononuclear cells has shown that there is a rapid rise in the proportion of T-lymphocytes which then goes back to normal by two weeks after the lesion begins. Evaluation of the macrophages soon after addition of the intratracheal ovalbumin has shown that these macrophages are producing a chemotactic factor for neutrophils and hence are likely responsible, at least in part, for the traffic of these inflammatory cells from blood to lung in the early stages of this lesion.

Significance to Biomedical Research and Institute Program: 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 complement 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 to Project: 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:

Szapiel, S.V., Elson, N.A., Fulmer, J.D., Hunninghake, G.W., and Crystal, R.G. Bleomycin-induced Interstitial Lung Disease in the Nude, Athymic Mouse. Am. Rev. Resp. Dis. (in press).

Gadek, J.E., Hunninghake, G.W., Zimmerman, R.L., and Crystal, R.G. Regulation of Release of Alveolar Macrophage Derived Neutrophil Chemotactic Factor. Am. Rev. Resp. Dis. (in press).

Hunninghake, G.W., Gadek, J.E., and Crystal, R.G. Human Alveolar Macrophage Neutrophil Chemotactic Factor: Stimuli and Partial Characterization (submitted).

Bernardo, J., Hunninghake, G.W., Gadek, J.E., Ferrans, V.J., and Crystal, R.G. Hypersensitivity Pneumonitis: Serial Changes in Lung Lymphocyte Subpopulations Following Exposure to Antigen (submitted).

Hunninghake, G.W., Gadek, J.E., Kawanami, O., Ferrans, V.J., and Crystal, R.G. Inflammatory and Immune Processes in the Human Lung in Health and Disease: Evaluation of Bronchoalveolar lavage. Am. J. Pathology (in press).

Hunninghake, G.W., Gadek, J.E., Szapiel, S.V., Strumpf, I.J., Kawanami, O., Ferrans, V.J., Keogh, B.A., and Crystal, R.G. The Human Alveolar Macrophage. Human Tissues and Cells in Biomedical Research (C. Harris, B.F. Trump, and G.D. Stoner, Eds). in Methods and Perspectives in Cell Biology, Academic Press, New York (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02407-05 PB

PERIOD COVERED

October 1, 1978 to September 30, 1979

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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Pulmonary Branch

SECTION

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NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
12.4	8.0	4.4

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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% 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, particularly the extracellular matrix. 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 likely reduces the ability of α1-antiproteinase to function normally. Therapeutic trials are ongoing for treatment of α1-antitrypsin deficiency using danazol therapy to increase release of the anti-protease from the liver and a direct replacement trial of α1-antiproteinase is ongoing.

925

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	V. Ferrans	Staff Investigator	NHLBI PA
	W. Roberts	Chief, Pathology Branch	NHLBI PA
	A. Fauci	Staff Investigator	I LCI
	R. Brady	Chief, Developmental and Metabolic Neutrology Branch	N DMN
	T. Waldman	Chief, Metabolism Branch	C MET

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 disease of know 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 disease 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 10% 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 trafficking to the lung, balanced by anti-proteases (i.e., αl-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 αl-antitrypsin deficiency (a hereditary disorder associated with panacinar emphysema), and various other clinical studies of lung diseases associated with destruction of the alveolar structures.

There are several forms of lung disease which have a known hereditary basis. By evaluating these "experiments in nature" we can derive an understanding of the structure and function of the lung which can then be applied

to more general understanding of pulmonary disorders. In addition to α1-antitrypsin deficiency discussed above, Fabry's Disease and hereditary disorders of connective tissue have also been studied.

Methods: Patients admitted to the Pulmonary Branch Clinical Service enter an extensive protocol which includes: detailed medical and pulmonary history and physical exam, routine serologic, roentgenographic and EKG studies, serologic studies aimed at immune processes; pulmonary function studies including lung volumes, flow rates, diffusing capacity, flow-volume curves, closing volume, closing capacity, body 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 thru 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) Detailed physiologic studies of patients with IPF have shown that it is very difficult to estimate the extent of inflammation and fibrosis in the lung using physiologic studies. The conventional physiologic parameter such as vital capacity, total lung capacity, diffusing capacity, and resting arterial blood gases have no relationship to the extent of disease as estimated by open lung biopsy. However, static deflation volume-pressure relationships do correlate with the degree of fibrosis as does the extent of drop of arterial oxygen tension with exercise. However, the latter is not specific for the fibrosis as it also correlates well with the extent of inflammation. In studies in collaboration with the Clinical Hematology Branch, NHLBI, the Pulmonary Branch has been evaluating conventional steady state exercise methodology with a newer breath by breath exercise protocol using an increasing amount of work load throughout the test. The latter has the advantage of being more rapid and in certain studies, noninvasive. In conjunction with the Critical Care Medicine Group, Clinical Center, the Pulmonary Branch has been evaluating the pulmonary circulation of patients with interstitial lung disease. Quite remarkably, their pulmonary hemodynamics are quite similar to patients with panacinar emphysema and different from those with chronic obstructive lung disease of the bronchitic variety. In the past year, methods have been developed to evaluate the work of breathing and to use this as a parameter of the extent of disease in these individuals. While preliminary studies suggest it is not very sensitive to the development of disease, it may prove useful for following response to drug therapy.

(2) Inflammatory and immune processes. As described in Project No. Z01 HL 02405-05 PB, a major development has been the adaptation of the fiber-optic bronchoscope to evaluate the inflammatory and immune processes of the lower respiratory tract in humans. This technique, however, relies on the assumption that the cells and noncellular components that are washed off the epithelial surface of the lower respiratory tract reflect those inflammatory and immune processes that are ongoing throughout the lung parenchyma. To test this hypothesis, methods have been developed to directly isolate inflammatory and immune effector cells directly from the lung of patients undergoing open lung biopsy for diagnostic purposes. These comparisons have shown that the lavage method gives an accurate reflection of the inflammatory and immune effector cells present and thus is a valid means to assessing individuals with these disorders. One of the most important accomplishments of the past year has been the characterization of the chronic inflammation in the lower respiratory tract of patients with interstitial lung disease using lavage and biopsy methods. Compared to normal individuals (see Project No. Z01 HL 02405-05 PB) patients with interstitial disease have increased numbers of inflammatory and immune effector cells in their lower respiratory tract. In addition, there are significant changes in the proportions of these cells present and such changes are likely the key to the pathogenesis of these diseases. Basically, the interstitial disorders that have been evaluated can be grouped into two categories of inflammation: a neutrophil type of inflammation and a lymphocyte type of inflammation. In the first, as typified by IPF, there is a chronic accumulation of neutrophils in the lower respiratory tract. Whereas normally, less than 1% of the inflammatory and immune effector cells present are neutrophils, in diseases like IPF, 5 to 25% of the effector cells are neutrophils. In contrast, in patients with sarcoidosis and hypersensitivity pneumonitis, there are no neutrophils but very high proportions of lymphocytes. Whereas normally, lymphocytes comprise less than 10% of the total inflammatory and immune effector cells, in these disorders lymphocytes comprise greater than 15% (often up to 50 to 60%) of the total cells present. Not only are there increased numbers of lymphocytes, but the relative proportions of types of lymphocytes change such that there are more T-lymphocytes. These T-lymphocytes often appear activated, i.e., increased numbers and receptors for the Fc portion of IgG and rosette with sheep red blood cells at 37°. As more interstitial disorders are evaluated, it is likely we will be able to characterize them on the basis of the inflammatory and immune effector cell subtypes that are present. These methods have also given insights into how the chronic inflammation is maintained. In idiopathic pulmonary fibrosis, studies of macrophages have shown that they have immune complexes on their surface and that these immune complexes induce the macrophages to release a chemotactic factor specific for neutrophils. This likely explains the mechanism by which neutrophils are chronically attracted to the lung in this disorder. In addition, while some of these patients have circulating immune complexes, a large proportion have immune complexes within the lower respiratory tract, suggesting that the immune complexes are being formed within the lung and not brought to the lung from elsewhere in the body. In addition, the use

of a reverse hemolytic plaque assay has demonstrated that large numbers of B-lymphocytes from the lungs of patients with idiopathic pulmonary fibrosis are making immunoglobulin compared to the B-lymphocytes of peripheral blood. Although the largest proportion of B-lymphocytes are producing IgG, significant numbers are also producing IgM or IgA. Whereas in peripheral blood, approximately $500/10^6$ lymphocytes are producing IgG, up to $30,000/10^6$ lymphocytes are producing IgG within the lungs of patients with IPF. Additional studies have demonstrated that the macrophages in these patients interact with neutrophils to induce the neutrophil to destroy lung fibroblasts. In contrast to idiopathic pulmonary fibrosis, the maintenance of the inflammation in sarcoidosis is likely mediated by the activated T-lymphocyte. These cells are producing a chemotactic factor for blood monocytes. This is of critical importance in a disease like sarcoidosis, as the attraction and activation of blood monocytes is important for the formation of granulomata which are characteristic of this disease.

(3) Morphology. In conjunction with the Pathology Branch, NHLBI, detailed studies have been ongoing as to the ultrastructure of the lung in health and disease. A new connective tissue structure, termed "anchoring fibrils" has been defined in the alveolar interstitium of patients with interstitial disease. These 200-600 angstrom wide, 4000-6000 angstrom long structures seem to insert into the basement membrane under sites of cuboidal epithelial cells in the regions of hemi-desmosomes. Similar structures are found in the tracheabronchial tree of the normal dog. In the latter circumstance, however, they are decreasing in numbers as one progresses down the tracheabronchial tree and are not found in the normal alveolar structures. Additional studies have shown that patients with interstitial lung disease have surprising nuclear inclusions which appear to be infoldings of the nuclear membrane. The reason for these are not known but are quite characteristic and found in up to 1% of the nuclei of the epithelial cells of the alveolar structures.

(4) Therapy. Current therapeutic protocols for the treatment of IPF include: (a) cyclophosphamide versus prednisone; (b) intramittent intravenous large bolus corticosteroids; and (c) dapsone, a sulfone used therapeutically to treat other disorders in which there seems to be an accumulation of neutrophils associated with immune complexes (as in the case of the lung in IPF). In sarcoidosis. current therapeutic protocols include: (a) the double blind use of intermittent high dose intravenous corticosteroids for a short period; and (b) the prospective evaluation of the prognostic significance of high intensity inflammation in the lung compared to low intensity inflammation in the lung with or without corticosteroid intervention.

(5) Smoking. Evaluation of alveolar macrophages recovered from smokers and nonsmokers have shown that the macrophages of smokers are spontaneously releasing a chemotactic factor for neutrophils. This likely explains why 2-3% of the inflammatory and immune effector cells of the

lower respiratory tract of smokers are neutrophils, whereas less than 1% are so in nonsmokers. In vitro studies of alveolar macrophages obtained from normal individuals have shown that, when exposed to cigarette smoke in culture dishes, normal alveolar macrophages will also produce this neutrophil chemotactic factor. It has been hypothesized, that in addition to having more neutrophils in their respiratory tract, that smokers are more at risk for lung destruction becasue their anti-proteases are not as effective as those of nonsmokers. To evaluate this hypothesis, the anti-elastase activity of components of lavage fluid of smokers was compared to nonsmokers for the effectiveness of the α1-anti-proteinase within the lower respiratory tract. Quite strikingly the α1-antiproteinase within the lavage fluid of the smokers was 50% as effective as an anti-elastase as was the material from nonsmokers. Thus it appears that the lungs of smokers have less protection against proteolytic attack than do the lungs of nonsmokers.

(6) Therapy. The hereditary disorder α1-antitrypsin deficiency is a disease in which those affected have a marked reduction of this serum glycoprotein by both functional and antigen criteria. Normally, α1-antitrypsin is the principle serum protease inhibitor. In the homozygous deficiency state (phenotype "ZZ"), 80% of affected adults have accelerated, usually fatal, destructive lung disease. Although considerable progress has been made in characterizing this protease inhibitor abnormality, clues to the precise pathophysiology and effective therapy for this disorder are lacking. Current indications suggest that the ZZ homozygous protein is a single amino acid substitution. Most likely, this abnormality results in changes in intracellular confirmation of the molecule such that sugar side chains cannot be added. Consequently the altered ZZ protein cannot be secreted in the normal fashion by the liver cells in which it is made. A drug trial with danazol, an "impeded" androgen which increases anti-protease release by the liver, has demonstrated that this drug will increase the levels of α1-antitrypsin in the serum of patients with α1-antitrypsin deficiency by 40%. Whether or not these increases will be efficacious for the therapy of this disease will require further detailed study. In addition, studies are ongoing to "cure" this disorder by direct replacement of the missing α1-antitrypsin. A protocol has been developed where partially purified α1-antitrypsin is given intravenously on a once a week basis for four weeks to patients homozygous with this disease. The purpose of the protocol is a pilot study to evaluate the feasibility of maintaining serum levels over a period of time in these patients. Initial indications suggest that with a once a week administration of the equivalent of the α1-anti-trypsin in two liters of plasma, "above threshold" levels can be maintained.

(7) Other studies have identified a new form of destructive lung disease not generally considered to be in this category. Evaluation of patients with acquired hypogammaglobulinemia have demonstrated that approximately 25% of patients with long standing immunoglobulin deficiency have a lower lobe emphysema by chest film. This lung destruction looks indistinguishable from those patients with α1-antitrypsin deficiency. In patients with acquired hypogammaglobulinemia, however, the lung destruction is likely secondary to repeated bouts of infection (as the primary pathogenic event)

initiating an increased protease burden and thus the protease anti-protease imbalance.

(8) Fabry's Disease, Angikeratoma Corporis Diffusion Universlae, is a sex-linked sphingolipid disorder. The lipid which accumulates is ceramide trihexoside and the deficient enzyme is ceramide trihoxosidase. Studies of Fabry's Disease has shown that there is abnormal lipid deposition in the airway epithelial cells. Correlates with functional abnormalities of the airways have shown that it likely is the basis for obstructive lung disease in these patients. In addition, correlation with smoking history has demonstrated that the patient with Fabry's Disease is greatly at risk if he smokes, since the airway obstruction seen with these patients is far beyond that attributable to smoking alone. Thus, it is likely that smoking thrusts these patients above a lowered threshold for the development of obstructive lung disease and suggests that Fabry's Disease could be added to the list of those genetic disorders associated with obstructive lung disease.

(9) Hereditary disorders of connective tissue. The connective tissue of lung is complex, consisting of four genetically distinct types of collagen, elastic fibers, seven types of glycosaminoglycans with their associated proteins and a variety of other macromolecules composing the ground substance. This complexity, together with the intricate anatomy of lung parenchyma, makes the study of the influence of connective tissue on the control of lung structure and function extraordinarily difficult. The major objectives of this proposal are attempts to use "experiments of nature" involving connective tissue (Marfan Syndrome, Ehlers-Danlos Syndrome, Osteogenesis Imperfecta) to help understand the role each connective tissue component plays in the maintenance of lung structure and function. The lung is the only organ of the body where it is relatively easy (using the sophisticated methodology of pulmonary function testing) to make direct inference on the role of connective tissue in controlling mechanical properties. Thus, the lung affords an opportunity for understanding the biochemical-mechanical correlates of connective tissue. This project is done in conjunction with biochemical studies on the control of synthesis and destruction of connective tissue (see Project No. ZO1 HL 02409-04 PB). Patients with specific connective tissue disorders are admitted to the Pulmonary Branch Clinical Service and pulmonary function tests are used to evaluate the "mechanical" status of their lungs. This is a long-term project but with methodology available today, it should be possible to specifically define the role of each connective tissue component in lung and demonstrate how these components describe pulmonary abnormalities in these hereditary disorders.

Significance to Biomedical Research and Institute Program

The interstitial 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 the control of synthesis and degradation of the extracellular matrix, we expect to make major inroads into understanding and treating these disorders.

Several of the studies outlined are long-term studies but are of fundamental significance in the understanding of the structure and function of lung in health and disease. The connective tissue of lung is so complex that it would be difficult to determine the role of each component of the interstitial connective tissue unless methods such as those described above can be utilized. As more patients are evaluated, patterns should emerge which help to determine the contributions of each component of connective tissue as they relate to pulmonary structure and function.

Proposed Course:

Studies as outlined will be continued. As methods are developed in the basic laboratory, they will be applied to study of 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 results of pharmacologic agents become promising, they will be studied in patients where applicable.

The αl-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 αl-antitrypsin above the threshold level by which these patients are more at risk. We are also doing preliminary studies to determine whether or not patients with αl-antitrypsin may be treated by the intravenous replacement of the deficient protein. There is no information available suggesting that the half-life of the normal protein is approximately 5 days. It may, therefore, be possible to treat patients with αl-antitrypsin deficiency by intravenous replacement in the same manner that hemophilia is treated.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02409-04 PB

PERIOD COVERED
October 1, 1978 to September 30, 1979

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

PI:	R. Crystal	Chief, Pulmonary Branch	NHLBI PB
Other:	P. Tolstoshev	Expert	NHLBI PB
	C. Boyd	Guest Worker	NHLBI PB
	L. Diaz de Leon	Guest Worker	NHLBI PB
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	B. Trapnell	Chemist	NHLBI PB
	K. Bradley	Chemist	NHLBI PB
	M. Schafer	Chemist	NHLBI PB
	J. Davidson	Staff Investigator	NHLBI PB
	S. Shibahara	Guest Worker	NHLBI PB
	K. Smith	Biological Laboratory Technician	NHLBI PB

COOPERATING UNITS (if any)

J. Moss	Staff Investigator	NHLBI CM
A. Nienhuis	Chief, Clinical Hematology Branch	NHLBI CL

LAB/BRANCH
Pulmonary Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
15.9	8.5	7.4

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The extracellular matrix of the alveolar structures is a critical determinant of lung structure and function in health and disease. Tissue culture studies have demonstrated a variety of lung cells both synthesize and destroy extracellular matrix complements. The fibroblasts synthesize collagen types I and III as well as producing a collagenase that destroys both collagen types. It also synthesized fibronectin, a glycoprotein important in cell-cell and cell-matrix interactions. The rate of collagen synthesis is rigidly controlled. However, there is environmental stimuli such as prostaglandins and mediators of activated lymphocytes can alter the differentiated state of fibroblasts with respect to collagen synthesis. The intracellular degradation of collagen has been defined: approximately one-third of all collagen synthesized by fibroblasts is destroyed within the cell prior to secretion. The portion of intracellular degradation can be modulated by altering the structure of collagen or by changing the levels of intracellular cyclic AMP. Studies of collagen gene structure have shown that the pro α2 gene contains significant numbers of noncoding sequences.

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Other:	G. Fells	Biologist	NHLBI PB
	I. Strumpf	Staff Investigator	NHLBI PB
	R. Zimmerman	Biologist	NHLBI PB
	G. Hunninghake	Staff Investigator	NHLBI PB
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	S. Rennard	Staff Investigator	NHLBI PB
Cooperating Units:			
	P. Kretschmer	Staff Investigator	NHLBI MH
	D. Wright	Staff Investigator	C PO
	G. Martin	Chief, Laboratory of Developmental Biology and Anomalies	D LDBA
	B. Steinman	Staff Investigator	D LDBA

Project Description:

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) fibronectin, a newly described class of glycoproteins involved in cell-cell and cell-matrix interactions. 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) Modulation of collagen production in cultured human lung fibroblasts. Methods have been developed to culture fibroblasts from human lung tissue of normal individuals as well as those with a variety of lung disorders. These fibroblasts have been extensively characterized and several are now maintained in the permanent collection of the American Type Culture Collection. These fibroblasts have been evaluated in terms of their ultrastructure as well as a variety of characteristics of their differentiated state. All the human lung fibroblasts studies to date produce both collagens I and III and no differences in the types of collagen produced have been detected in fibroblasts cultured from patients with various interstitial lung disorders.

(2) Evaluation of collagen production in human fetal lung of fibroblasts has demonstrated that they produce the same quantity of collagen throughout their growth curve and as well as over at least 25 population doublings. This invariance of collagen production suggests the production of the macromolecule is tightly controlled as befits its critical importance in maintaining lung structure and function.

(3) Evaluation of cyclic AMP levels in human lung fibroblasts has demonstrated that this cyclic nucleotide is critically linked to collagen production. Agents that increase intracellular cyclic AMP (e.g., isoproternol, PEG_1, cholera toxin) all decrease collagen production by these fibroblasts. Other β-stimulents such as norepinephrine and epinephrine also cause this phenomena. The concept has been developed that under normal conditions fibroblasts of the body are generally "suppressed" in terms of their potential for collagen production by endogenous β-stimulents. This suggests that the β-adrenergic system "holds back" collagen production and thus has the potential for significantly modifying the quantity of extracellular matrix components. This also leads to possible mechanism of β-agonist-induced fibrosis, i.e., when propanolol is given to a susceptible individual, this β-blocker prevents the

normal suppression of collagen production by endogenous β-agonists and thus increases the production of collagen in certain organs.

(4) It is clear now that a significant proportion of collagen produced by fibroblasts is degraded within the cell prior to secretion. This appears to be a potent control mechanism for modulating the amounts of intact collagen that are produced by the cells. Studies with proline analogs (e.g., azedetine, cis-hydroxyproline) cause collagen to be defective, resulting in a marked increase in intracellular degradation. "Defective" collagen can also be produced in lung fibroblasts by omitting ascorbic acid, a cofactor for the normal hydroxylation of proline residues, a post-translational mechanism that is critical for normal collagen helical structure. Without ascorbic acid these cells produce proportionally increased amounts of defective collagen and intracellular collagen degradation increases 2 to 3 fold. Studies have also shown that at least part of the modulation of collagen production by fibroblasts by cyclic AMP is modulated through this intracellular degradative process. Intracellular collagen degradation appears to occur, at least in part, in lysozomes, as studies with lysozomal inhibitors indicate that the intracellular degradation of defective collagen can be markedly inhibited. Additional studies of fibroblasts from individuals with osteogenesis imperfecta have shown that intracellular collagen degradation is the same in these cells as in those from normal individuals.

(5) Studies of collagen production in tendon, skin, and lung of sheep of varying gestational age have demonstrated that messenger RNA levels significantly control the production of collagen by these tissues. The percentage of protein synthesis devoted to collagen production varies in these tissues as a function of gestational age during fetal development, and appears to be paralleled, in general, by collagen messenger RNA levels. The exception is in skin, where high messenger RNA levels are maintained late in gestational life whereas the tissue appears not to be using all the messenger RNA that is present.

(6) Studies are in progress concerning the Ehlers-Danlos IV syndrome, an hereditary disorder of connective tissue in which type III collagen is not made. Messenger RNA for collagen can be isolated from fibroblasts from these patients and are being compared to normal fibroblasts. To search for the underlying genetic defect in this disorder, studies are utilizing these fibroblasts to isolate messenger RNA for collagen types I and III and to evaluate type I and type III collagen gene structure.

(7) Studies are ongoing to evaluate the production of elastin by a variety of tissues including lung explants and smooth muscle cultures. It is clear

that elastin is produced as a 70,000 dalton product. Peptide maps have been developed to evaluate the structure of this protein and compare it from tissue to tissue. In addition, an elastin messenger RNA has been prepared as has an elastin complementary DNA. These components are currently being used to evaluate elastin gene structure and the control of elastin biosynthesis.

(8) Detailed studies are ongoing to evaluate the control of collagen production at the gene level. Using the techniques of recombinent DNA, approximately 50% of the sheep pro $\alpha 2$ gene has been isolated. Whereas the coding sequences for the 3' 50% of the pro $\alpha 2$ gene is approximately 2,500 bases, restriction map studies have shown that this structural information is spread out over at least 14,000 bases. Mapping of this genomic DNA using electron microscopic techniques has demonstrated that there are at least 12 intervening, non-coding sequences spread out through this 14,000 base region. Detailed restriction maps have been prepared and are being used to evaluate the fine structure of this gene together with DNA sequencing of selected regions. Studies are continuing to isolate the genes for other major connective tissue components found in lung and these are being evaluated using similar technology.

(9) Studies of connective tissue destruction have concentrated primarily on evaluating the sensitivity of fibronectin to proteases released from the peripheral blood neutrophil. Fibronectin, a critical connective tissue component that appears to be important in cell-cell and cell-matrix interactions, is exquisitely sensitive to the neutral proteases of the neutrophil suggesting a mechanism by which neutrophils in the lung may significantly derange lung structure by attacking an important connective tissue matrix component.

(10) Studies of collagenase in the human peripheral blood neutrophil have shown that it is present in both the primary and secondary granules. These sites appear to harbor different collagenases as the collagenase in the primary granule attacks both collagen types I and III whereas the collagenase in the secondary granule attacks type I only. In addition, these collagenases appear to have different inhibitory spectra, molecule weights and relative states of activation.

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 arrangement 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 contol 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 are involved in destructive processes of the extracellular matrix and the interstitial lung disorders are involved primarily with abnormalities in 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 of these disorders.

Proposed Course to Project: Our studies will coninue 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 subcellular levels.

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ANNUAL REPORT OF THE
MOLECULAR DISEASE BRANCH
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
OCTOBER 1, 1978 THROUGH SEPTEMBER 30, 1979

The overall objective of the research program of the Molecular Disease Branch is the ascertainment of the molecular properties and physiological importance of the human plasma apolipoproteins and lipoproteins, the determination of the mechanisms involved in the regulation of cellular cholesterol biosynthesis 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 processes involved in the biosynthesis, transport, and catabolism of plasma apolipoproteins-lipoproteins. Within this framework the plasma lipoproteins are viewed as a polydisperse collection of lipoprotein particles, the apolipoprotein composition of which is determined by the laws of mass action. The unique constituent of the plasma lipoprotein is the apolipoprotein moiety and the distribution of the plasma apolipoproteins is governed by the relative concentration 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 degradation in normal subjects and in patients with dyslipoproteinemia and atherosclerosis.

Prerequisite to our understanding of the physiological role of plasma apolipoproteins in lipid metabolism is a detailed knowledge of the chemistry and structure of the human plasma apolipoproteins. We have undertaken a detailed characterization of the human apolipoproteins and have recently extended these studies to the D apolipoprotein. ApoD has been isolated in homogeneous form by affinity chromatographic techniques. In addition to the monomeric form, a large molecular weight form of apoD was isolated and characterized. Detailed analysis revealed that this larger protein was a disulfide dimer of apoD and monomer apoA-II. The apoD-A-II dimer was present in all plasma samples analysed. ApoD-A-II is therefore similar to the apoE-A-II complex previously reported by other investigators. It now appears that apoA-II interacts with other plasma apolipoproteins which contain cysteine/cystine residues forming covalent disulfide complexes. The physiological importance of these oligomeric proteins remains to be delineated. ApoD has become of major importance since it was recognized to be the cholesterol ester exchange protein which transfers cholesterol esters between plasma lipoproteins.

Studies have also continued during the year on apoE, the apolipoprotein which is elevated in patients with dysbetalipoproteinemia (Type III hyperlipoproteinemia), and in animals and man fed a high cholesterol diet. ApoE has been isolated in homogeneous form from normal subjects and patients with Type I, III, and V hyperlipidemia. The amino acid composition and molecular weight

of the E apolipoprotein are similar from all patients. Isoelectric focusing of the purified E apolipoprotein revealed that patients with Type III hyperlipoproteinemia were missing the polymorphic band designated as E_3. Patients with Type V hyperlipoproteinemia, like Type III patients, have elevated plasma levels of apoE, however, the isoelectric focusing pattern of these patients was similar to controls. The significance of the E_3 deficiency in patients with dysbetalipoproteinemia, and the physico-chemical basis for the polymorphism of the E apolipoprotein is currently being investigated.

An additional apolipoprotein, apoG, has been purified to homogeneity from the plasma of normal individuals and Type V patients as well as from thoracic duct lymph. This apolipoprotein has a unique amino acid composition, molecular weight, and plasma distribution. Detailed studies are underway to determine if this apolipoprotein has any homology with apolipoproteins of other species, including rat apoA-IV. The physiological importance of this apolipoprotein has not as yet been determined.

A systematic analysis of the molecular properties of the human apolipoproteins continues to be an active area of investigation 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. An understanding of 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 in our Branch have demonstrated that the human apolipoproteins are a unique class of proteins. Apolipoproteins A-II, A-I, and C-I have been shown to self-associate. The association scheme for the individual apolipoproteins is as follows: apoA-I, monomer-dimer-tetramer-octomer; apoA-II, monomer-dimer; apoC-I, monomer-dimer-trimer. Of particular importance was the recognition that concomitant with self-association was a major increase in apolipoprotein structure. In the monomer form, apolipoproteins were nearly devoid of organized structure, whereas in the oligomeric form the conformation increased to that characteristic of globular proteins. These dramatic changes in structure are greater than those reported for any other self-associating protein. The driving force for the dramatic change in conformation of the apolipoproteins is the shielding of nonpolar residues from the solvent and is thus hydrophobic in nature.

The self-association of plasma apolipoproteins has been shown to be extremely sensitive to various ligands, pH, ionic strength, and protein concentration. Of particular note has been the demonstration of the effects of hydrostatic pressure on the molecular properties of apolipoproteins. In interacting systems when there is a major change in solvent exposure of the nonpolar residues during oligomer formation there may be a significant change in molar volume. This is primarily due to the unfavorable interaction between nonpolar solutes and water. Presumably decreases in the amount of "ordered water" allow nonpolar solutes to expand when shielded from solvent. The mode of association of apoA-I, apoA-II, and apoC-I was shown to be significantly effected by pressure and the volume changes on association are of the greatest magnitude reported in the literature. The major changes in molecular

properties of apolipoproteins as a function of pressure reemphasize the unique physico-chemical properties of these apolipoproteins.

The quaternary structure of plasma lipoproteins is as yet incompletely understood. Of particular significance in the quaternary structure may be the presence of heterogeneous ("mixed") apolipoprotein associations. The association of apolipoproteins with other proteins including enzymes such as lipoprotein lipase and lecithin cholesterol acyltransferase also undoubtedly play a major role in the transport and metabolism of plasma lipoproteins. A detailed study of the heterogeneous association of apolipoproteins in aqueous solution has been initiated. These studies have focused on apoA-I - apoA-II and apoA-II - apoC-I interactions. To facilitate the investigation of "mixed" apolipoprotein interactions, one of the apolipoproteins, apoA-II, was nitrated with tetranitromethane, the adduct of which has characteristic spectral properties which permits direct analysis of the interaction of the apolipoproteins. This technique was used in conjunction with analytical ultracentrifugation, and circular dichroism to evaluate the "mixed" interactions of the apolipoproteins. A strong interaction between the individual apolipoproteins was observed, and discrete specific oligomeric species were formed. The "mixed" oligomers formed were a one-to-one molar ratio of apoA-I and apoA-II and two molecules of apoA-II to four molecules of apoC-I. These studies were interpreted to indicate that homogeneous as well as heterogeneous interactions are of high affinity and result in the formation of very specific homogeneous and heterogeneous oligomeric complexes. These homogeneous and heterogeneous complexes are undoubtedly of fundamental importance in the organization of the quaternary structure of plasma lipoproteins.

During the last several years the cellular binding, uptake, and catabolism of plasma lipoproteins has attracted a great deal of interest. Investigation of the role of cholesterol in the atherogenic process has focused primarily on the etiological importance of plasma lipoproteins and cellular receptor sites with less emphasis on the possible role played by defects in intracellular cholesterol metabolism. A key enzyme in the cellular metabolism of lipoprotein cholesterol is acid cholesterol esterase, the lysosomal enzyme responsible for hydrolysis of cholesterol esters into cholesterol and free fatty acids.

Acid cholesterol esterase (ACE) activity was assayed in lymphocytes of patients (N=36, aged 25-45) with angiographically documented coronary artery disease (CAD) and patients of similar age with structural heart disease. All known risk factors were evaluated in the patients. ACE activity was significantly lower ($p<.005$) in the patients with premature cardiovascular disease. Two other risk factors, low-HDL cholesterol and smoking, were also statistically different between the two groups. However, multivariant analysis showed the reduction in ACE in the CAD group to be significant ($p<.005$), and independent of the incidence of the other known risk factors. A reduction in ACE activity would be proposed to play an important role in the atherogenic process by reducing cholesterol ester hydrolysis and decreasing the formation of free cholesterol subsequently available for removal from the cell. Based on these results, we have proposed that a deficiency in lysosomal ACE activity

may represent a new, independent risk factor for the development of 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 staff of the Molecular Disease Branch has extensively analyzed the factors involved in the modulation of the enzymic activity of HMG-CoA reductase. HMG-CoA reductase has been isolated in electrophoretically homogeneous form from chicken and rat liver. HMG-CoA reductase was shown to be present in an active-inactive form. Utilizing radiolabeled ATP, and a monospecific antibody prepared against HMG-CoA reductase, it was 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 phosphorylaton 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 [^{32}P]-ATP, phosphoprotein phosphatase, and a partially purified cytosolic protein kinase designated reductase kinase kinase have established that reductase kinase undergoes reversible phosphorylation-dephosphorylation. These results provided evidence that the activity of both HMG-CoA reductase as well as reductase kinase are modulated by a phosphorylation-dephosphorylation reaction sequence. The active form of HMG-CoA reductase is dephosphorylated, whereas the phosphorylated form of reductase kinase is active. The regulation of HMG-CoA reductase by a bicyclic cascade is similar to bicyclic cascade systems previously reported reported for glutamine synthetase and phosphorylase. Modulation of the enzymic activity of HMG-CoA reductase by the bicyclic cascade mechanism provides a rapid short term method for the regulation of the pathway for cholesterol biosynthesis. The elucidation of this new mechanism for the modulation of the enzymic activity of HMG-CoA reductase provides new insights into the regulation of cellular cholesterol biosynthesis.

The synthesis, transport, and catabolism of plasma lipoproteins continues to be an active area of investigation within the Branch. The focus on the site of synthesis of apolipoproteins has changed from predominantly an analysis of the apolipoproteins secreted by the liver to an analysis of the role of the intestine in apolipoprotein synthesis. A new technique utilizing the immunoperoxidase procedure has been adapted for analysis of apolipoproteins synthesized by the intestine in normal subjects and patients with dyslipoproteinemia. Normal human jejunal tissue was analyzed by the immunoperoxidase technique for the presence of apoA-I, apoA-II, and apoB. All three apolipoproteins were shown to be present in intestinal epithelial cells with the maximum staining present at the apical region of the villous. Intestinal tissue of patients with Tangier Disease, which are characterized by low plasma levels of apolipoproteins A-I and A-II, were also analyzed. ApoA-I, apoA-II, and apoB were all shown to be present in the intestinal tissue of patients with Tangier Disease. The presence of both apoA-I and apoA-II in Tangier Disease is consistent with our previous metabolic studies

in these patients which demonstrated that the low plasma levels of apoA-I and apoA-II were due to increased catabolism rather than defective synthesis. The synthesis of apoA-I and apoA-II is relatively normal and the major defect in these patients appears to be due to accelerated apolipoprotein A-I and A-II catabolism resulting in the virtual absence of high density lipoproteins.

Patients with abetalipoproteinemia were also investigated and apoA-I and apoA-II were shown to be synthesized by the intestine. Abetalipoproteinemic patients, despite their absence of apoB containing lipoproteins including chylomicrons, VLDL and LDL, are still able to absorb some dietary fat. These lipids appear to be transported from the intestine in apoA-I and apoA-II containing lipoproteins. An additional study was undertaken in these patients to document intestine absorption of dietary fats and vitamins. In normal subjects vitamin A is incorporated into chylomicrons and transported to the liver where it is combined with its carry protein for plasma transport. Patients with abetalipoproteinemia were also shown to absorb and transport vitamin A but within HDL. These combined results give new insight into the function and metabolism of plasma lipoproteins in patients with abetalipoproteinemia.

The intestinal transport of plasma lipoproteins has also been assessed utilizing lymph obtained from an indwelling thoracic duct cannula in patients undergoing treatment for renal disease. Subjects have been studied both before and after successful renal transplant. Thoracic duct lymph lipoproteins were characterized with respect to particle size, hydrated density, and apolipoprotein composition. Lymph lipoproteins were all spherical and no discoidal or "nascent" lipoproteins were observed. The majority of the lipoproteins were of a hydrated density <1.006 g/ml, however HDL were also present. Lymph HDL were larger (Stokes diameter: 9.2-12.7nm) and of lower density (peak $F^{o}_{1.20}$ 3.5-5.0) than plasma HDL from the same subject (Stokes diameter: 3.5-11.5 nm, peak $F^{o}_{1.20}$ 2.2-2.9). The triglyceride/cholesterol ratio of lymph HDL was 5-7 times that of plasma HDL for each subject studied.

The role of the intestine in the production of apoA-I and apoA-II was also addressed in these patients. The daily transport of apolipoproteins A-I and A-II from the thoracic duct into the plasma was 40-70 percent greater than their predicted plasma synthesis rates. These results provide evidence for the recirculation of apoA-I and apoA-II between plasma and thoracic duct lymph. These studies indicate that the lipoproteins within lymph and the recirculation of apolipoproteins between lymph and plasma are of major importance in our ultimate understanding of the pathways for lipoprotein synthesis, transport, and catabolism.

An evaluation of the polydispersity of plasma lipoproteins continues to be a productive area of research. Interest has focused on HDL since the recognition that HDL-cholesterol was a negative risk factor for the development of premature cardiovascular disease. A simplified, two-step preparative ultracentrifugation procedure has been developed to isolate the three major density fractions of HDL (HDL_{2b}, d 1.063-1.100 g/ml; HDL_{2a}, d 1.100-1.130 g/ml; HDL_3, d 1.130-1.210 g/ml). The concentration of both the apolipoprotein and lipid

constituents were variable within HDL_{2b}, and HDL_{2a}, but essentially constant in HDL_3 in the fasting plasma of twelve normolipidemic subjects. The HDL_{2b} and HDL_{2a} subfractions were directly related to the total HDL concentration, while HDL_3 was relatively constant and not significantly related to total HDL content. ApoA-I and apoA-II were present in all subfractions however apoE and apoB were found primarily within HDL_{2b}. These results confirm the heterogeneity of HDL and the importance of detailed analysis of subfractions as studies are directed toward the elucidation of the structure and function of HDL.

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. Apolipoproteins are now known to serve as cofactors for enzymes (apoC-II and lipoprotein lipase; apoA-I and lecithin cholesterol acyl transferase), ligands for receptor binding (apoB and apoE), and exchange proteins (cholesterol ester exchange - apoD). The quantitation of apolipoproteins represent a major prerequisite to the analysis of apolipoprotein concentration and metabolism in normal individuals and patients with dyslipoproteinemia. Immunochemical assays employing radial immunodiffusion or rocket electrophoresis have been developed in the Branch for several of the apolipoproteins including apoA-I, apoA-II, apoC-II, and apoB. Assays currently under development include apoE, apoD, apoC-I, apoC-III, and apoG. Quantification of apolipoproteins in normal individuals and patients with disorders of lipid metabolism and atherosclerosis are presently in progress.

The metabolism of plasma lipoproteins continues to be actively investigated within the Branch. The metabolism of apoA-I and apoA-II, the two major protein moieties of HDL have been analyzed. Studies with radiolabled apoA-I and apoA-II were performed in 14 normal subjects, 3 patients with Type I hyperlipidemia, and two patients with abetalipoproteinemia. The plasma residence times in normal subjects was 4.46 days for apoA-I and 4.97 days for apoA-II. ApoA-I is, therefore, catabolized at a slightly faster rate than apoA-II ($p<.005$) in plasma. Compartmental models were constructed separately for apoA-I and apoA-II utilizing plasma and urine data. The model for apoA-I contained two plasma compartments, one decaying faster than the other. Only a single plasma compartment was required for the apoA-II compartmental model. The extra plasma compartment of apoA-I was found to account for the decrease in apoA-I residence time. These data are also interpreted as indicating that not all apoA-I and apoA-II are on the same particles within HDL and catabolized as a unit.

Patients with familial lipoprotein lipase deficiency (Type I) have very low levels of HDL, however, these patients are not prone to premature cardiovascular disease. Studies with radiolabeled apoA-I and apoA-II revealed a relatively normal synthesis rate of apoA-I and apoA-II, however a markedly enhanced catabolism (plasma, residence time of 2.45 days and 2.68 days for apoA-I and apoA-II respectively). The lowered plasma levels of HDL in these patients appears, therefore, to be due to accelerated catabolism rather then defective synthesis. The lack of premature cardiovascular disease in these patients remains an interesting clinical enigma.

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Abetalipoproteinemia is a disease, as outlined above, which is characterized by the lack of all apoB containing lipoproteins, e.g., chylomicrons, VLDL, and LDL, as well as decreased HDL. These patients have a constellation of clinical findings including neurological defects, malabsorption, and anemia, however no premature cardiovascular disease. Studies with radiolabeled apoA-I and apoA-II indicated that the decreased level of HDL apolipoproteins is due to increased catabolism (residence time apoA-I and apoA-I, 2.52 and 2.43 days) with relatively normal synthesis. HDL represents the sole major class of plasma lipoproteins in these patients, and their lack of cardiovascular disease is of particular interest.

Studies have also been initiated on the metabolism of the C-II and C-III apolipoproteins. The metabolism of the C apolipoproteins is of major importance since apoC-II is the cofactor for lipoprotein lipase, the enzyme involved in the catabolism of triglyceride rich lipoproteins. ApoC-II and apoC-III studies were performed in 7 normal volunteers. The fractional catabolic rate of apoC-III was greater than apoC-II. The shape of the apoC-II and apoC-III decay curves was markedly different in VLDL when compared to HDL. These findings indicate that apoC-II and apoC-III are not metabolized as a unit on a lipoprotein particle that the metabolism of these apolipoproteins may be different in separate lipoprotein density classes. The studies on C apolipoprotein metabolism will be continued in normal subjects and extended to patients with disorders of triglyceride metabolism.

The metabolism of triglycerides has been studied in 59 normal or hypertriglyceridemic subjects employing radiolabeled glycerol. Analysis by a compartmental model revealed two pathways for the synthesis of VLDL-triglycerides from glycerol. Kinetic curves for VLDL-triglyceride were analyzed in normal subjects who were of ideal body weight, mildly obese, or markedly obese. Markedly obese subjects where shown to have increased synthesis rates for VLDL triglycerides. The markedly obese subjects with hypertriglyceridemia had relatively normal fractional catabolic rates while the normotriglyceridemic markedly obese patients had an increased fractional catabolic rate resulting in essentially normal plasma triglyceride concentrations. Future studies will now be directed toward the analysis of the coordinate metabolism of apolipoproteins and triglycerides in normal subjects, and patients with dyslipoproteinemia and atherosclerosis.

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PERIOD COVERED
October 1, 1978 through September 30, 1979

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
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.8	1.8	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)

Research in our laboratory has focused on the composition and metabolism of plasma lipoproteins (apo) in normal and dyslipoproteinemic man. Over the past 12 months we have completed studies utilizing radiolabeled high density lipoproteins (HDL), apoA-I, and apoA-II in 10 normals, 3 patients with type I hyperlipoproteinemia, and 2 patients with abetalipoproteinemia. The interaction of radiolabeled apoA-I and apoA-II with plasma lipoproteins, particularly HDL subfractions, has been of special interest. The 24 hour output and lipoprotein distribution of apoA-I and apoA-II in human thoracic duct lymph has also been characterized in six individuals undergoing thoracic duct drainage. Studies utilizing radiolabeled very low density lipoproteins (VLDL), apoC-II, and apoC-III$_2$ have been completed in 7 normal subjects. Methodology for studying radiolabeled apoE kinetics in man has been developed. Ongoing computer analysis of data obtained in normal and dyslipoproteinemia subjects is nearing completion.

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PHS-6040
(Rev. 10-76)

Project Description

Objectives:

1) To complete studies on apoA-I and apoA-II metabolism in normal and dyslipoproteinemic man.

2) To elucidate the interrelationship between lymph lipoproteins and plasma lipoproteins in man.

3) To define the plasma kinetics of apoC-II, apoC-III_2, and apoE in normal and dyslipoproteinemic subjects.

4) To determine the effects of various diets and drugs on plasma lipoprotein composition in man.

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 are in the process of reimplementing a computerized record keeping system for purposes of storing and analyzing clinical and lipoprotein data on our patients.

Major Findings:

1) Findings based on radiolabeled apoA-I and apoA-II plasma kinetics are presented in the following table. All values represent means.

	Normals (n=14)	Tangier (n=3)	Type I (n=3)	Abetalipoproteinemia (n=2)
Plasma A-I*	129	2.0	82	44
Plasma A-II*	25	2.6	24	23
A-I SR**	11.53	3.68	13.24	9.01
A-II SR**	2.11	1.32	4.29	1.72
A-I RT***	4.46	.23	2.45	2.52
A-II RT ***	4.97	.90	2.68	2.43

* plasma apoprotein levels are given in mg %.
** SR is synthesis rate in mg/kg/day.
*** RT is residence time in plasma in days.

When radiolabeled HDL was used in 8 subjects, plasma residence times were 4.23 days for apoA-I and 4.82 days for apoA-II. These data indicate that apoA-I is catabolized at a significantly faster rate than apoA-II ($p<.005$) in plasma. Patients with homozygous familial HDL deficiency (Tangier disease) have decreased apoA-I and apoA-II synthesis, and an increased catabolism of

these apolipoproteins. Patients with type I hyperlipoproteinemia and abetalipoproteinemia have enhanced catabolism of both apoA-I and apoA-II.

2) Analysis of 24 hour output and lipoprotein distribution in human thoracic duct lymph of apoA-I and apoA-II indicates that the transport of both of these apolipoproteins exceeds predicted synthesis by 40-70%. Approximately 25% of apoA-I and 20% of apoA-II was associated with chylomicrons and VLDL in lymph indicating that these lymph lipoproteins may be an important source of plasma HDL apoA-I and apoA-II. The data also suggest that HDL is readily transferred from plasma to lymph accounting for the greater than predicted synthesis of apoA-I and apoA-II. Previous studies from our laboratory have indicated that lymph chylomicron apoA-I and apoA-II can serve as precursors for these constituents within HDL.

3) ApoC-II and apoC-III kinetics were studied in 7 normals. The mean plasma residence times for apoC-II were 1.13 days and for apoC-III were 0.90 days. The decay of these apoproteins within VLDL was different from that within the VLDL and HDL density fractions.

4) Patients with all forms of hypertriglyceridemia (type I, IV, V hyperlipoproteinemia) have decreased HDL cholesterol levels but these levels do not increase when such patients are placed on type I, type IV or type V diets. The level of HDL cholesterol in normal subjects and type V patients increase when these individuals are placed on nicotinic acid. Esterogen administration in normal subjects increases HDL cholesterol. HDL cholesterol levels are inversely correlated with body mass index [weight in kg/(height in meters)^2].

Significance to Biomedical Research and the Program of the Institute:

HDL cholesterol has been shown to be inversely correlated with the incidence of coronary artery disease (CAD), while LDL cholesterol has been positively correlated with CAD. Factors regulating the concentration of LDL and HDL protein and lipid are therefore of great interest. The data discussed above provide insights into the regulation of HDL metabolism.

Proposed Course:

Continuing objectives include study of apolipoprotein metabolism (apoA-I, apoA-II, apoB, apoC-I, apoC-II, apoC-III, apoE) in normal and dyslipoproteinemic subjects. These studies are of fundamental importance in our understanding of lipid transport and metabolism in normal individuals, and in patients with disorders of lipid metabolism and atherosclerosis.

Publications:

1. Schaefer, E. J., Eisenberg, S., Levy, R. I.: Lipoprotein apoprotein metabolism. J. Lipid Res. 19: 667-687, 1978.

2. Schaefer, E. J., Blum, C. B., Levy, R. I., Jenkins, L. L., Alaupovic, P., Foster, D. M., Brewer, H. B., Jr.: Metabolism of high density

lipoprotein apolipoproteins in Tangier disease. New Eng. J. Med. 299: 905-910, 1978.

3. Schaefer, E. J., Levy, R. I.: Composition and metabolism of high density lipoproteins. In Lipoprotein Metabolism. Eisenberg, S. (ed.), Prog. Biochem. Pharmacol. S. Karger, Basel. Vol. 15, p. 186-201, 1979.

4. Schaefer, E. J., Levy, R. I., Anderson, D. W., Danner, R. N., Brewer, H. B., Jr., Blackwelder, W. C.: Plasma triglycerides in regulation of HDL-cholesterol levels. Lancet 2: 391-393, 1978.

5. Schaefer, E. J., Foster, D. M., Jenkins, L. L., Lindgren, F. T., Berman, M., Levy, R. I., Brewer, H. B., Jr.: The composition and metabolism of high density lipoprotein subfractions. Lipids 14: 511-522, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02010-08 MDB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Structure and Function 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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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.3	1.3	3.0

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

ApoD has been isolated in electrophoretically homogeneous form from HDL by affinity and gel permeation chromatography. ApoD contains carbohydrate and several disulfide bridges by amino acid analysis. A higher molecular weight form of apoD was also isolated, and shown to be a apoD-A-II dimer. ApoD has recently been proposed to be the cholesterol ester exchange protein which is involved in the exchange of cholesterol ester between plasma lipoproteins.

ApoE has been isolated in electrophoretically homogeneous form from normal subjects and patients with Type III and Type V hyperlipoproteinemia. The amino acid composition of apoE from all subjects was similar by amino acid analysis. On isoelectric focusing apoE from Type III patients was missing the E_3 band.

ApoG, a newly discovered apolipoprotein, was purified from thoracic duct lymph and plasma of patients with Type V hyperlipidemia. The apolipoprotein has a unique amino acid composition, is a glycoprotein and contains several disulfide bridges. The physiological role of apoG on lipoprotein metabolism is as yet unknown.

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Project Description

Objective:

1) Isolation and characterization of apoD.

Methods Employed

The methods for the isolation of plasma apolipoproteins have been detailed in previous reports. Our overall objective has been the development of affinity chromatographic procedures for the isolation of apolipoproteins since these methods are rapid, and have a high yield. Our initial studies on the isolation of apoD involved the fractionation of apoHDL on hydroxyapatite. A small quantity of highly purified apoD was obtained, and an antibody to apoD was prepared in goats. Subsequent large scale purification of apoD has been performed employing an anti-apoD affinity column, followed by fractionation on hydroxyapatite.

Major Findings:

ApoD purified by the procedure was electrophoretically homogeneous on disc gel electrophoresis in urea, and in sodium dodecyl sulfate. The isolated apolipoprotein contains carbohydrate, and several cystine residues by amino acid analysis. During characterization of apoD, a higher molecular weight form of apoD was isolated. Detailed chemical characterization of this form of apoD revealed it to be a disulfide dimer of apoD and apoA-II. Antibodies to both apoD and apoA-II reacted with apoD-A-II, reduction and carboxymethylation yielded apoD and monomeric apoA-II, and sodium dodecyl sulfate electrophoresis in DTT revealed two protein bands corresponding to apoD and apoA-II (monomer). ApoD and apoD-A-II have been shown to be present in all samples of apoHDL thus far analyzed. These studies are interpreted as indicating that apoA-II becomes covalently linked with apoD sometime prior to delipidation of HDL, however, it is as yet unclear if this apoD-A-II protein is present in plasma prior to the isolation of the plasma lipoproteins.

Objective:

2) Isolation and characterization of apoE.

Methods Employed:

As outlined above, the isolation of the plasma apolipoproteins has been facilitated by the use of affinity chromatography. ApoE was isolated from delipidated chylomicrons-VLDL (plasma density < 1.006 g/ml) isolated by heparin-sepharose chromatography from normal subjects, and patients with Type III and I hyperlipoproteinemia.

Major Findings:

ApoE has been isolated in electrophoretic homogeneous form by affinity chromatography. The apolipoprotein has a molecular weight of approximately 32,000. ApoE can be isolated in two major structural forms which includes the apoE monomer, and a dimer of approximately 64,000. The dimer is readily converted to the monomer on electrophoresis in sodium dodecylsulfate and dithiothreitol. A very small quantity of apoE-A-II has been isolated from chylomicrons-VLDL.

The chemical properties of apoE have been compared from normal subjects and patients with Type III and V hyperlipidemia. The amino acid composition and molecular weight of apoE were similar in all samples isolated. On isoelectric focusing, apoE from normal subjects, and patients with Type V hyperlipidemia were similar with three major and two minor bands. Purified apoE from patients with Type III hyperlipidemia were missing the electrofocusing band which corresponded to the E_3 band of apoE isolated from normal subjects.

The reason for the polymorphism of apoE on isoelectric focusing is as yet unknown. No change was observed following treatment with neuraminidase. The molecular mechanism which is responsible for the absence of E_3 in Type III hyperlipoproteinemia is as yet unknown. The deficiency of E_3 is not now considered to be unique to the Type III phenotype, and it has been observed in subjects without Type III. These latter subjects are usually hypocholesterolemic individuals. The physiological significance of the E_3 difficiency is yet to be determined.

Objective:

3) Isolation and characterization of apoG.

Methods:

ApoG was isolated from human thoracic duct lymph and from lipoproteins of density < 1.006 g/ml of normal subjects or patients with Type V hyperlipidemia by affinity chromatography on heparin followed by sephacyl 200 gel permeation chromatography.

Major Findings:

ApoG was isolated in electrophoretically homogeneous form from human thoracic duct lymph and from VLDL-chylomicrons of normal subjects and patients with Type V hyperlipoproteinemia. The apoprotein was approximately 50,000 in molecular weight by gel electrophoresis in sodium dodecyl sulfate. The amino acid composition of apoG from all sources was similar. This apolipoprotein appears to be synthesized by the intestine, and to be predominately associated with triglyceride rich lipoproteins. Little apoG was associated with LDL or HDL. A detailed comparison of the physico-chemical

properties of apoG with all other known human plasma apolipoproteins and rat apoA-IV are underway in order to determine if apoG is a new apolipoprotein. The physiological role of apoG in lipoprotein structure and metabolism is as yet unknown.

Objective:

4) Determination of the site of phosphorylation of glycogen synthetase catalyzed by cAMP independent protein kinase and phosphorylase b kinase.

Methods Employed:

Native glycogen synthetase and glycogen synthetase phosphorylated by a cAMP-independent protein kinase or phosphorylase b kinase were prepared by Drs. Joseph Larner and Peter Roach, University of Virginia.

Major Findings:

Glygogen synthetase phosphorylated with phosphrylase b kinase was subjected to automated Edman degradation on the Beckman sequencer. The major site of phosphorylation was serine at position 7 in the amino terminal region of the sequence. Sequence analysis of glycogen synthetase phosphorylated with a cAMP-independent protein kinase also revealed the major site of synthesis to be serine position 7. These results indicate that both kinases are phosphorylating the same site on glycogen synthetase and would be expected to, therefore, have the same physiological effect. Current studies are underway to determine the site of phosphorylation on glycogen synthetase catalyzed by other kinases.

Objective:

5) Development of improved techniques for the automated sequence analysis of peptides and proteins.

Methods Employed:

Modification of the Beckman 890B Sequencer including the use of a large reaction cup and cold trap were detailed in last year's annual report.

Major Findings:

Our preliminary results using a large reaction cup (75 percent increase in surface area) were described in last year's report. These studies have been extended and the large cup has been shown to be a major improvement in automated sequence design. Sequence analysis of myoglobin and bovine parathyroid hormone clearly demonstrated that degradation with the modified sequencer had less overlap and non-specific cleavage than degradations with the commercial instrument. Beckman Instruments (Palo Alto, CA) has now made these two modifications available as modification units for the Beckman Sequencer to the general biomedical research community.

Significance to Biomedical Research and the Program of the Institute:

The isolation, characterization, and sequence analysis of the plasma apolipoproteins is an ongoing program within our laboratory designed to ultimately elucidate the mechanisms involved in the synthesis, transport, and metabolism of the plasma triglycerides and cholesterol in normal individuals and patients with disorders of lipid transport and atherosclerosis.

Proposed Course:

The isolation, characterization, and structural analysis of the major apolipoproteins in intestinal lymph and plasma will be continued. A major emphasis will continue on apoC-II, D, E, and G. These apolipoproteins will be isolated and characterized from normal individuals as well as patients with dyslipoproteinemia. These studies will provide further insight into the role of the apolipoproteins in the structure and physiological role of this unique group of plasma lipoproteins.

Publications:

1. Thomas, F., and Brewer, H. B., Jr.: Automated Edman degradations: studies with a large sequencer cup and high speed drive. Anal. Biochem. (in press).

2. Brewer, H. B., Jr., Schaefer, E., Zech, L., and Osborne, J. C., Jr.: Proceedings of the High Density Lipoprotein Methodology Workshop. DHEW Publication (in press).

3. Brewer, H. B., Jr., Schaefer, E., Osborne, J. C., Jr., and Zech, L.: Lipoproteins: structure, function, and metabolism. Proceedings of International Symposium on New Aspects in Diagnosis and Treatment of Hyperlipidemia (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02011-04 MDB

PERIOD COVERED
October 1, 1978 through September 31, 1979

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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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.2	2.2	4.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The apolipoprotein composition of plasma lipoproteins is governed not only by the composition of the nacent lipid-apolipoprotein complex, but also depends critically upon the concentration and composition of other components of plasma, including other plasma lipoproteins. The distribution of the majority of apolipoproteins among plasma lipoproteins is governed by the laws of mass action and can in many cases be treated in terms of equilibrium constants. The redistribution of apolipoproteins with metabolism can be viewed in terms of perturbations on these equilibrium constants. This work is directed towards a greater understanding of the molecular forces involved in the quaternary organization of plasma lipoproteins. In order to quantitate these interactions we have investigated the solution properties of apolipoproteins. These studies have been extended to include an analysis of the molecular properties of modified apolipoproteins and also quantitation of "mixed" interactions between apolipoproteins in aqueous solution. These "mixed" interactions are of high affinity and result in formation of specific mixed oligomers. The oligomers (homogeneous and heterogeneous) of apolipoproteins in plasma are undoubtedly of major importance in the quaternary organization of plasma lipoproteins.

960

PHS-6040
(Rev. 10-76)

Project Description

Objective:

1) Evaluation of the role of apolipoproteins in the quaternary organization of HDL. Previous reports have summarized in detail the molecular properties of apoA-I and apoA-II, the major protein components of HDL. Briefly, both of these apolipoproteins have little secondary and tertiary structure in their monomeric forms and both self-associate with concomitant major changes in secondary, tertiary, and quaternary structure. The driving force for both systems is the shielding of nonpolar residues from solvent and is thus hydrophobic in nature. Apolipoproteins also undergo specific "mixed" interactions with one another. Quantitative analysis of these interactions by classical techniques with the native apolipoproteins is however quite complex. For instance, mixtures of apoA-I and apoA-II contain a minimum of six species in solution (monomers and dimers of apoA-II, and monomers, dimers, tetramers, and octamers of apoA-I) plus any mixed oligomers. One way to overcome these difficulties is to modify the spectral properties of one of the apolipoproteins; we have chosen to nitrate the tyrosine residues of apoA-II by reaction with tetranitromethane. The absorption spectrum of the resulting derivative is "red shifted" by approximately 100 nm from that of the native species. The molecular properties of nitrated apoA-II resemble the native species in many ways: 1) nitrated apoA-II cross-reacts with antibodies made to native apoA-II; 2) both apolipoproteins self-associate according to a monomer-dimer scheme; 3) both apolipoproteins undergo concomitant major changes in secondary structure with self association. This derivative, along with the native species, has been used to evaluate the molecular interactions between apoA-I and apoA-II.

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:

The concentration versus radius profile of nitrated apoA-II at equilibrium in the Model E ultracentrifuge in the presence and absence of apoA-I was obtained by absorbancy mesurements at 381 nm. The absorption spectrum of nitrated apoA-II is sensitive to the degree of ionization of the nitrotyrosine residues, with an isosbestic point at 381 nm. Data obtained at this wavelength do not depend upon changes in the degree of ionization of nitrotyrosine residues due to mixed or self-association. Since only those species

that contain nitro-apoA-II will contribute to data obtained at 381 nm, complex formation between apoA-I and apoA-II is detected easily at this wavelength. The data obtained in the presence and absence of apoA-I were analysed statistically according to the following equation:

$$C(r) = C_{10}(m)e^{D_{10}M_{10}(r^2-m^2)} + K_{20}C_{10}(m)^2 e^{D_{20}M_{20}(r^2-m^2)} + \sum_{\substack{i=1 \\ j=1}}^{n} \alpha_{ij}\, C_{10}(m)^i e^{D_{ij}M_{ij}(r^2-m^2)}$$

where:

c(r) is the total concentration of species containing nitrated apoA-II at position r in the cell; $c_{10}(m)$ is the concentration of nitrated apoA-II at the meniscus; K_{20} is the dimerization constant for nitrated apoA-II; M_{10} and M_{20} correspond to the monomer and dimer molecular weights of nitrated apoA-II, 17,740 and 35,480 respectively; $D = \omega^2 (1-\bar{v}\rho)/2RT$, ω is the angular velocity, $\bar{v}$ is partial specific volume, ρ is the solvent density, R is the gas constant and T is the absolute temperature; the subscripts i and j correspond to the number of subunits of species i and j in the mixed complex and

$$\alpha_{ij} = K_{ij}\,(C_{01}(m))\,\frac{\varepsilon_{ij}}{\varepsilon_{10}} \text{ where}$$

K_{ij} is the equilibrium constant for mixed complex formation, $C_{01}(m)$ is the concentration of apoA-I at the meniscus, ε_{ij} is the extinction coefficient of species i; and ε_{10} is the extinction coefficient for nitro-apoA-II.

The interaction between apoA-II and apoA-I in aqueous solution was also demonstrated experimentally by fluorescence techniques. Since the absorption spectrum of tryptophan overlaps the emission spectrum of tyrosine, electronic energy transfer can occur between these two chromophors; this transfer depends upon the reciprocal of the sixth power of the distance between donor and acceptor residues. ApoA-II contains eight tyrosine and no tryptophan residues and apoA-II contains four tryptophan residues per mole of protein; complex formation should result in an increase in the intensity of tryptophanyl fluorescence.

Energy transfer between apoA-II and apoA-I was found to be quite sensitive to the concentrations of apoA-I and apoA-II. At low concentrations of both apoproteins (~0.1 mg/ml) increases in fluorescence intensity (333 nm) as high as 100% were observed. With dilution, the increase in fluorescence intensity increased dramatically at all concentrations and ratios investigated. The

absorption spectrum of nitro-apoA-II overlaps the emission spectrum of apoA-I and thus a quenching of tryptophanyl fluorescence would be expected upon complexation with this derivative. This result was observed and the quenching was found to be a saturable phenomenon and paralleled the results obtained with native apoA-II summarized above.

These combined results are interpreted to indicate a strong interaction between apolipoproteins A-I and A-II in aqueous solution. The mixed interaction is specific for complexes containing an equimolar ratio of apoA-I to apoA-II and the equilibrium constant for mixed interactions is greater than the corresponding equilibrium constants for self-association.

Objective:

2) Evaluation of the effect of pressure on the molecular properties of apolipoproteins, lipoproteins, and lipoprotein particles. In interacting systems where the degree of exposure of nonpolar residues changes with oligomer formation, one usually observes a corresponding change in molar volume. This is primarily due to the unfavorable interaction between nonpolar solutes and water. Presumably decreases in the amount of "ordered water" allow nonpolar solutes to expand when shielded from solvent. Apolipoproteins A-I and A-II undergo major conformational changes that are concomitant with self-association, the oligomers being more highly structured than the corresponding protomers. With these systems the partial specific volume of the oligomers is greater than that of the protomers and thus these systems are sensitive to pressure; the oligomers dissociating with increasing pressure according to the equation, $\partial \ln K/(\partial P)_t = -\Delta v/RT$; where P is the hydrostatic pressure, R is the gas constant, K is equilibrium constant, T the absolute temperature and $\Delta\gamma$ is the change in partial specific volume upon complexation. The effects of pressure are presently being extended to the apoC-I system.

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 a Spinco Model E ultracentrifuge and a Chromatrix Laser Light Scattering Photometer. Calculations and least square fits of the data to various models were performed with the MLAB program on the DEC system 10 computer at NIH.

Major Findings:

The effects of pressure on interacting systems depends upon the molar volume change, and thus is detected most easily in systems where there is a large change in molecular weight and/or the degree of exposure of nonpolar moieties upon complexation. The molecular weight of monomeric apoC-I is

only 6630, however with association there is a large increase in secondary structure and presumably a large change in the degree of exposure of non-polar groups. In previous studies at 30,000 rpm in the Model E ultracentrifuge the molecular weight of apoC-I ranged from 8,000 to 16,500. These data when analysed according to modes of association were most consistent with a monomer-dimer-trimer scheme, however, monomer-trimer and monomer-dimer-tetramer models gave comparable fits and with these data alone one could not distinguish between these three modes of interaction. Based on our results with the apoA-I and apoA-II systems, sedimentation equilibrium experiments were performed at lower rotor speeds and with shorter solution heights; both of these changes should favor the oligomeric species. Higher apparent weight average (M_w^{app}) moleccular weights were obtained under the new conditions, and the dependence of M_w^{app} upon rotor speed was similar to that observed previously with the apoA-I and apoA-II systems, i.e., increasing M_w^{app} at any given concentration of protein with decreasing rotor speed. A least squares analysis of the data still gave comparable fits to the monomer-dimer-trimer and monomer-dimer tetramer modes of interaction. The change in partial specific volume upon interaction required to account for the data was however the same for both models, 0.027 ml/mg. Thus the apoC-I system is comparable to apoA-I and apoA-II; each system is quite sensitive to pressure with major changes in partial specific volumes upon complexation. Additional experiments at higher protein concentrations will be required to distinguish between the monomer-dimer-trimer and monomer-dimer-tetramer modes of interaction. We are presently evaluating the use of absorption measurements above 300 nm in the Model E ultracentrifuge, for M_w^{app} data at high protein concentrations.

Objective:

3) Evaluation of the mechanism of VLDL-HDL exchange of the C-apolipoproteins in the quaternary organization of VLDL and HDL. In the metabolic conversion of VLDL to LDL, apolipoproteins C-I, C-II, and C-III are released and can be isolated in HDL. The molecular mechanism responsible for the distribution of apolipoproteins during lipolysis is not known, but may well involve lipid free species. The exchange of apolipoproteins between plasma lipoproteins can in many ways be viewed as an equilibrium between apolipoproteins (lipid free) and lipoprotein particles in which the corresponding equilibrium constants change with lipolysis. In order to quantitate this type of interaction we have undertaken a systematic investigation of the solution properties of apolipoproteins. We have summarized in previous reports the molecular properties of apoC-I and apoC-III in aqueous solution. Briefly, both of these apolipoproteins self-associate, the former undergoing major conformational changes whereas the secondary structure of the latter changes very little with oligomer formation. With a knowledge of the parameters governing the self-association of these apolipoproteins, one can begin to evaluate quantitatively the "mixed" interactions among apolipoproteins. Over the last year the mixed association between apoC-I and apoA-II has been evaluated.

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:

The analysis of the mixed association between two self-associating proteins is simplified greatly if the spectral properties of one of the proteins is modified by nitration of the tyrosine residues with tetranitromethane. As indicated in (1) above this modification results in a "red shift" of the absorption spectrum well into the visible range. Sedimentation.equilibrium measurements of nitrated apoA-II were performed in the presence and absence of apoC-I at several rotor speeds. The equilibrium profiles of concentration versus distance from the center of rotation were obtained at 280 nm, where all species in solution contribute to the data, and at 381 nm where only those species containing nitro-apoA-II contribute, i.e., monomers and dimers of nitrated apoA-II and any mixed oligomers. The profiles obtained were consistent with a strong interaction between apoC-I and nitrated apoA-II. The combined data were most consistent with the presence of mixed oligomers containing two molecules of nitrated apoA-II and four molecules of apoC-I. This result is especially intriguing since apoA-II has been shown previously to self-associate according to a monomer-dimer scheme. As indicated in (2) above, the presence of trimers or tetramers of apoC-I in solution is still under active investigation. If tetramers are shown to exist, then the present data would be interpreted to indicate that a specific mixed interaction between the oligomers of apoC-I and those of apoA-II exists in aqueous solution.

Objective

4) Evaluation of the molecular properties of radiolabled apolipoproteins.

The in vivo metabolism of plasma lipoproteins has been investigated by several laboratories using iodine labeled plasma lipoproteins. In the majority of these studies various density fractions of plasma have been labeled directly; since the majority, if not all, of the density fractions of plasma are heterogeneous with respect to apolipoprotein composition, several apolipoproteins are simultaneously labeled with this procedure. The distribution of most apolipoproteins changes dramatically with metabolism, those initially labeled in one

density fraction of plasma usually become unequally divided between several density classes, and the fate of a given apolipoprotein is quite difficult to follow quantitatively. More recently, apolipoproteins have been labeled in their lipid-free form, and then incorporated into plasma lipoproteins by *in vitro* or *in vivo* incubation with plasma.

The corresponding kinetics of *in vivo* metabolism are of course dependent directly upon any perturbations in the molecular properties of the plasma lipoproteins caused by the labeling and/or incubation procedure employed; the labeled apolipoprotein must mimic the distribution of the corresponding unlabeled species at each stage of metabolism. Since the molecular properties of several apolipoproteins have been reported, perturbations in the properties of labeled apolipoproteins can be evaluated experimentally.

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:

Apolipoproteins A-I and A-II were labeled with ^{125}I and ^{131}I respectively according to the procedure described by MacFarlane. The efficiency of labeling was 61% for apoA-I and 49% for apoA-II, resulting in specific activities of 106 μCi/mg and 82 μCi/mg respectively. Unbound iodine was removed by dialysis; less than 1% of the radioactivity of the final sample was soluble in trichloroacetic acid. Radiolabeled apolipoproteins were diluted with native apolipoproteins (labeled: unlabeled was 1.56×10^{-3} for apoA-I and 1.2×10^{-2} for apoA-II) and analyzed by permeation chromatography through sephadex G-150 superfine (90 x 1.4 cm) resin. The elution profiles were monitored by absorbancy and radioactivity measurements. Both apolipoproteins A-I and A-II self-associate and their column elution profiles are distinct and sensitive to changes in the mode of self-association. [^{131}I] Apo-II eluted as a single asymmetrical peak, consistent with a monomer-dimer association scheme reported previously. The elution profile of [^{125}I] Apo-AI was much more complex, as expected for a system that self-associates according to a monomer-dimer-tetramer octamer scheme. The elution profile of both systems, particularly apoA-I, is quite sensitive to initial protein concentrations, due to the reversible protomer-oligomer equilibria; the elution profile of the faster migrating component decreases with increasing protein concentration.

Thus if the radiolabeled species did not participate in this reversible interaction with the unlabeled species, the profiles obtained by absorbancy would be quite different than those obtained by radioactivity measurements, since the initial concentrations of labeled apolipoproteins were much lower than the corresponding concentrations of unlabeled species. The elution profiles for labeled and unlabeled species were however quite similar for both apolipoproteins. This result is interpreted to indicate that the labeled species interact with the unlabeled species in a reversible manner consistent with a monomer-dimer scheme for apoA-II and with a monomer-dimertetramer octamer scheme for apoA-I.

The elution profile as obtained by absorbancy measurements however preceded slightly than that obtained by mesurements of radioactivity for each apolipoprotein. This is most probably due to a non-specific interaction between the iodine labeled apolipoprotein and the column matrix, although some more specific perturbation in the molecular properties of the apolipoproteins could not be ruled out. We are presently extending the comparison of iodine labeled and native apolipoproteins to sedimentation equilibrium and circular dichroic measurements.

Significance to Biomedical Research and Program of the Institute:

This work is directed toward a greater understanding of the forces involved in the molecular organization and function of the plasma lipoproteins. The plasma lipoproteins are quite complex and their structure is sensitive to numerous perturbations, including pressure, ionic strength, temperature, and the concentration 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:

During the next year the studies concerning the self and mixed interactions between apolipoproteins will be continued, with special emphasis on mixed interactions between the A and C apolipoproteins. In addition, the studies comparing the molecular properties of native and modified apolipoproteins will be expanded to include nitrated apoA-I and iodinated apoC-III and apoE. These studies are forming a firm framework for our ultimate understanding of *in vivo* plasma lipoprotein interactions.

Publications

1. Osborne, J. C., Jr.: Evaluation of volume changes in associating systems by sedimentation equilibrium. J. Biol. Chem. 253: 359-360.

2. Osborne, J. C., Jr., Powell, Grace M., and Brewer, H. Bryan, Jr.: Analysis of the mixed association between human apoA-I and apoA-II in aqueous solution. Biochem. Biophys. Acta (in press)

3. Lloyd, M. A., Osborne, J. C., Jr., Safer, B., Huff, G. and Merrick, W. C.: Characteristics of eukaryotic initiation factor 2 and its subunits. J. Biol. Chem. (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02012-04 MDB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Isolation, Characterization, and Regulation of Reductase Kinase and HMG-CoA Reductase

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.	Visiting Scientist	MDB	NHLBI
Other:	John 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, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.1	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)

Rat and chicken HMG-CoA reductase activity can be modulated in vitro by a phosphorylation-dephosphorylation reaction sequence. A microsomal reductase kinase from liver catalyzes the phosphorylation of HMG-CoA reductase and histones. Histone and HMG-CoA reductase phosphorylation was enhanced by cyclic AMP. Stimulation of phosphorylation of cAMP was blocked by protein kinase inhibitor.

Incubation of reductase kinase with phosphoprotein phosphatase resulted in a time-dependent decrease in the ability of reductase kinase to catalyze the phosphorylation of histones and HMG-CoA reductase. Incubation of phosphoprotein phosphatase inactivated reductase kinase with [γ ^{32}P]-ATP plus Mg^{+2} and a partially purified protein kinase designated reductase kinase kinase resulted in a parallel increase in [^{32}P] protein-bound radioactivity and decrease in the ability to inactivate HMG-CoA reductase. Incubation of [^{32}P] labeled reductase kinase with phosphoprotein phosphatase resulted in a time-dependent loss of protein-bound radioactivity and decrease in the ability to inactivate HMG-CoA reductase. Polyacrylamide gel electrophoresis of [^{32}P] reductase kinase revealed that all radioactivity and enzyme activity was located in a single band.

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PHS-6040
(Rev. 10-76)

Project Description

Objective:

1) Purification and characterization of rat liver HMG-CoA reductase.

Methods Employed:

Microsomal pellets from rat livers were isolated by ultracentrifugation. HMG-CoA reductase was solubilized by a freeze-thaw technique developed in this laboratory. As previously described, the solubilized enzyme was fractionated with ammonium sulfate (35-50% saturation), and the supernatant heated to 65°C for 10 min. The sample was centrifuged and the supernatant applied to a coenzyme A and HMG-CoA affinity column.

Major Findings:

Rat liver HMG-CoA reductase was purified to homogeneity within 16 hrs with high recovery. The specific activity of the enzyme was 10-15,000 nmoles of mevalonate formed per min per mg protein. The purified enzyme migrated as a single protein band on aqueous and SDS-acrylamide gel electrophoresis and had a monomeric and oligomeric molecular weight of 50,000 and 200,000 daltons respectively. The purified enzyme had a Km of 1 μm for HMG-CoA. An antibody to purified rat HMG-CoA reductase was prepared in goats. The specificity of goat antiserum prepared against the purified enzyme was documented by double diffusion analysis and quantitative precipitin reactions with the solubilized and microsomal enzyme.

Objective:

2) Purification and characterization of microsomal HMG-CoA reductase kinase.

Methods Employed:

The microsomal HMG-CoA reductase kinase which catalyzes the phosphorylation of HMG-CoA reductase has been purified to homogeneity utilizing DEAE-cellulose, phosphocellulose chromatography, gel filtration (sepharose 6B) absorption chromatography on alumina C_γ and thin layer isoelectric focusing (pH 4-6.5). All preparations of reductase kinase were tested for kinase activity utilizing histone as a substrate.

Major Findings:

Rat liver microsomal reductase kinase has been purified to homogeneity. Purified reductase kinase migrated as a single band on aqueous and SDS-gel electrophoresis, and had a monomeric and oligomeric molecular weight of 60,000 and 380,000 daltons respectivley. On isoelectric focusing a pI of 5.6 ± 0.2 was obtained.

Solubilized and partially purified reductase kinase catalyzed the phosphorylation of histone II-A. This phosphorylation was stimulated 2-3 fold by cAMP. However, purified reductase kinase preparations failed to show any stimulation of histone phosphorylation by cAMP. These results suggest that there are two reductase kinases, one is cAMP independent and the other is cAMP dependent. Current studies are underway to separate and characterize the cAMP dependent reductase kinase.

Objective:

3) Phosphorylation-dephosphorylation of rat liver HMG-CoA reductase kinase and HMG-CoA reductase.

Methods Employed:

Microsomal, solubilized and purifed HMG-CoA reductase can be phosphorylated (inactivated) by ATP+Mg^{2+} and a microsomal reductase kinase. Microsomal reductase kinase also exists in active and inactive forms. Incubation of reductase kinase with phosphoprotein phosphatase resulted in the loss of reductase kinase activity. Phosphorylation of inactivated reductase kinase with ATP+Mg^{2+} and a protein kinase designated reductase kinase kinase resulted in reactivation of reductase kinase.

Major Findings:

Microsomal HMG-CoA reductase is inactivated (phosphorylated) by ATP+Mg^{2+} and a protein kinase designated reductase kinase. Phosphorylated HMG-CoA reductase can be dephosphorylated (reactivated) with phosphoprotein phosphatase. Reductase kinase also exists in interconvertible active and inactive forms. Incubation of reductase kianse with phosphoprotein phosphatase resulted in a time-dependent decrease in the ability of reductase kinase to catalyze the phosphorylation of histones and inactivate HMG-CoA reductase. Incubation of phosphoprotein phosphatase inactivated reductase kinase with [γ-^{32}P]-ATP plus Mg^{2+} and a partially purified protein kinase designated reductase kinase kinase resulted in a parallel increase in [^{32}P] protein-bound radioactivity and ability to decrease the activity of HMG-CoA reductase. Incubation of [^{32}P] labeled reductase kinase with phosphoprotein phosphatase resulted in a time-dependent loss of [^{32}P] protein-bound radioactivity and decrease in the ability to inactivate HMG-CoA reductase. Polyacrylamide gel electrophoresis of purified reductase kinase incubated with reductase kinase kinase and [γ-^{32}P]-ATP plus Mg^{2+} revealed that the [^{32}P] radioactivity and reductase kinase enzymic activity were located in a single electrophoretic position. Dephosphorylation of [^{32}P] labeled purified reductase kinase with phosphoprotein phosphatase was associated with significant loss of radioactivity and enzymic activity in the protein band ascribed to reductase kinase. These results provide evidence that the activity of reductase kinase like HMG-CoA reductase is modulated by a reversible phosphorylation-dephosphorylation reaction sequence.

Significance to Biomedical Research and the Program of the Institute:

The above described studies are directed toward an understanding of the short-term regulation of the rate limiting enzyme of cholesterol biosynthesis, HMG-CoA reductase. An elucidation of the mode of control of this enzyme will permit a detailed analysis of the factors involved in the cellular regulation of cholesterol metabolism in cells of normal subjects, and patients with atherosclerosis.

Proposed Course:

A systematic investigation of properties and modulation of HMG-CoA reductase and reductase kinase will be continued. The cAMP-dependent reductase kinase and reductase kinase kinase will be purified to homogeneity and their role in the regulation of HMG-CoA reductase activity studied. Both *in vitro* and *in vivo* regulation of HMG-CoA reductase and reductase kinase will be investigated during the next year. These studies will enhance our understanding of the role of this pivotal enzyme in the regulation of cholesterol synthesis.

Publications:

1. Beg, Z. H., Stonik, J. A., Brewer, H. B., Jr.: 3-hydroxy-3-methylglutaryl coenzyme A reductase: regulation of enzymatic activity phosphorylation and dephosphorylation. Proc. Natl. Acad. Sci. USA. 75: 3678-3682, 1978.

2. Beg, Z. H., Stonik, J. A., and Brewer, H. B., Jr.: 3-hydroxy-3-methylglutaryl coenzyme A reductase from avian liver: catalytic properties. Biochim. Biophys. Acta. 572: 83-94, 1979.

3. Beg, Z. H., Stonik, J. A., and Brewer, H. B., Jr.: Characterization and regulation of reductase kinase, a cAMP independent protein kinase which modulates the activity of HMG-CoA reductase. Proc. Natl. Acad. Sci. USA (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02014-03 MDB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Human Plasma Lipoproteins and Apolipoproteins: Isolation and Quantitation by Immunochemistry

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:
3.25	.25	3.0

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Free and total cholesterol has been quantitated in fractionated thoracic duct lymph, HDL subfractions HDL_{2a}, HDL_{2b}, and HDL_3 using the micromethod developed in our laboratory. Cholesterol analyzer sensitivity has been improved so that as little as 1 mg/dl of free cholesterol can be quantitated enzymically.

The normal ranges for C-II and LpB have been established by radial immunodiffusion. Normal ranges for A-I and A-II apolipoproteins have been remeasured. A-I immunoquantitation has been studied by rate nephelometry for protein-protein interactions

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PHS-6040
(Rev. 10-76)

Project Description

1) Objective:

To quantitate free and total cholesterol within thoracic duct lymph and its lipoprotein subfractions and within HDL_{2b}, HDL_{2a}, and HDL_3.

Methods Employed:

The Beckman enzymic-electrode cholesterol analyzer (as previously modified and evaluated in this laboratory) was standardized using 20-50 ul of a previously quantitated free and total cholesterol control sample. The system was evaluated for accuracy, sensitivity and linearity.

Major Findings:

Under the assay conditions samples could be quantitated accurately and with a sensitivity of 1 mg/dl for both free and total cholesterol. Due to the unique nature of thoracic duct lymph lipoproteins no deviation from linearity was seen with highly lipemic samples. The sensitivity of the analyzer made possible the quantitation of free and total cholesterol within HDL_{2b}, HDL_{2a}, and HDL_3 and the sum of these parts correlated excellently with the free and total cholesterol assayed within total HDL.

2) Objective:

The preparation of antibodies for use in quantitating the apolipoproteins, rat HMG-CoA reductase, and the lipoproteins.

Methods Employed:

100-400 μg of pure antigen were injected into two years or older goats with complete or incomplete Freund's adjuvant. Plasma was collected by plasmaphoresis. All plasmas were purified by precipitation of their IgG fraction, dialysis, and concentration by ultrafiltration to an appropriate antibody titer.

Major Findings:

Monospecific IgG fractions were obtained for apolipoproteins AI, AII, CI, CII, CIII, D, E, lipoprotein LpB, lipoprotein particles of LDL and HDL and the enzyme HMG-CoA reductase. Antibody titers were measured by double diffusion and radial immunodiffusion. Each antibody has been utilized in RID, EIA, double diffusion and crossed immunoelectrophoresis techniques. Because the apolipoproteins are imbedded within lipoprotein particles it was found necessary to organically delipidate all samples if pure antigens were used as standards. TMU, dodecyl sulfate, or heat delipidation techniques have been found inadequate.

3) Objective:

To quantitate the apolipoprotein C-II and the lipoprotein particle LpB. To determine recoveries for the apolipoproteins following organic delipidation.

Methods Employed:

Single radial immunodiffusion (RID), "rocket" immunoelectrophoresis (EIA) and double diffusion assays have been used to identify and quantitate apoC-II and LpB.

Major Findings:

A preliminary normal range for apoC-II has been determined on 36 normolipemic plasmas. The mean ± 1 SD was 3.1 ± 1.4 mg/dl. The normal range for LpB as determined by EIA was 95.5 ± 26.0 mg/dl. The recovery after organic delipidation for AI, AII, and CII were 104, 97, and 100% respectively.

4) Objective:

To quantitate apoA-I by rate nephelometry.

Methods Employed:

The Beckman ICS rate nephelometer was used in a modified manual mode. Standard nephelometric buffers and reagents were utilized.

Major Findings:

The apoA-I values obtained by rate nephelometry did not correlate well with quantitation by RID or EIA. The addition of albumin, A-II, and whole plasma proteins diminishes the rate of reaction when compared to pure A-I. Since A-I is known to self-associate and has been shown to be associated with A-II by cross-linking experiments, we feel that the loss of rate in the presence of other proteins is a result of protein-protein associations.

5) Objective:

To quantitate the fatty acids in the media and cell culture of MCF-7 cells from human breast cancer.

Methods Employed:

Gas chromatography was performed using glass columns packed with 10% SP-2340 in a Beckman GC-65 gas chromatograph. Temperature programming was used to complete elution of the fatty acids C_6-C_{22}.

All cell culture samples were saponified and the free fatty acids extracted into hexane. Methylation was performed in 0.5 ml reaction vials

by mixing 50 ul of extract with 50 ul of 0.2 M (3% w/v) trimethylanilinium hydroxide in methanol. The reaction mixture was heated at 55°C for 3 minutes before injection, or equal volumes of sample and TMAH were injected into the 315°C flash heater. Percentage of methylation (>96%) was monitored in all samples through the added internal standard (IS), tridecanoic acid.

Quantitation was performed by comparison of peak areas to pure methylated fatty acids and to the pure fatty acids after methylation by TMAH.

Major Findings:

Insulin has been shown to stimulate fatty acid synthesis in a number of tissues by a variety of mechanisms. One of these mechanisms involves an increase in the activity of the first enzyme in the de novo fatty acid synthetic pathway, acetyl CoA carboxylase.

MCF-7, a human breast cancer cell line in long term culture, responds to insulin with increased lipid synthesis. Differential substrate studies as well as direct measurement of enzyme activities suggest that the enzyme activity in fatty acid synthesis regulated by insulin is acetyl CoA carboxylase.

Insulin stimulation of fatty acid synthesis in MCF-7 human breast cancer cells has been proposed to be mediated by increased acetyl CoA carboxylase activity. Since the lipids synthesized in response to insulin are predominantly the long chain polar lipids found in membranes (20), the lipogenic effect of insulin may be related to its overall mitogenic action on these cells. Thus, modulation of acetyl CoA carboxylase activity by insulin may be linked to regulation of cell growth.

Significance to Biomedical Research and the Program of the Institute:

The increased sensitivity of the cholesterol analyzer for quantitating free and total cholesterol will make possible the monitoring of these entities within the even narrower density ranges presently important in lipid research. Subfractionated HDL cholesterol for the study of premature CAD, and subfractionated thoracic duct lymph cholesterol for the study of apolipoprotein synthesis and recirculation have been quantitated.

The quantitation of all the apolipoproteins is important. The definition of normal ranges within plasma and the normal distribution of the apolipoproteins within the lipoprotein particles is a prerequisite for our understanding of metabolism in normal and patients with dyslipoproteinemia.

Proposed Course:

The continued development of quantitative immunochemical methods for the apolipoproteins will require further purification of antigens and the

production, isolation, and characterization of monospecific antibodies. Apolipoprotein quantitation will be of major importance in studies of the metabolism of lipoproteins in normal subjects and subjects with disorders of lipoprotein metabolism and atherosclerosis.

Publications:

1. Bronzert, T. J.: Cholesterol quantitation with the Beckman Oxygen Electrode-Enzymic Analyzer. Proceedings of the High Density Lipoprotein Methodology Workshop. DHEW Pub. (in press).

2. Bronzert, T. J.: Quantitation of cholesterol within fractionated plasma lipoproteins. Test of the Month 4, No. 4 (1978).

3. Anderson, D. A., Bronzert, T. J., Schaefer, E. J., Niblack, G. D., Zech, L. A., Lindgren, F. T., Forte, T., Brewer, H. B., Jr.: Transport of apolipoproteins A-I and A-II from human thoracic duct lymph into plasma. J. Lipid Res. (in press).

4. Monaco, M. E., Osborne, C. K., Bronzert, T. J., Kidwell, W. R., Lippman, M. E.: The mechanism of insulin regulation of lipid synthesis in MCF-7 human breast cancer cells. European Journal of Biochemistry (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02015-02 MDB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Isolation, Characterization, and Quantitation of Lipoproteins and Apolipoproteins from the Plasma and Lymph of Normal Individuals and Patients with Dyslipoproteinemia

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

PI:	David Anderson, Ph.D.	Staff Fellow	MDB	NHLBI
Other:	Ernst Schaefer, M.D.	Staff Associate	MDB	NHLBI
	H. Bryan Brewer, Jr., M.D.	Chief	MDB	NHLBI

COOPERATING UNITS (if any) Dr. E. N. Gerasimova, Myasnikov Institute, Moscow, USSR; Dr. A. V. Nichols, Donner Laboratory, University of California, Berkeley, CA; Dr. F. T. Lindgren, Donner Laboratory, University of California, Berkeley, CA

LAB/BRANCH
Molecular Disease Branch

SECTION
Peptide Chemistry

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.6	

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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)

A simplified, two-step preparative ultracentrifugation procedure was developed to isolate three density fractions of human plasma high density lipoproteins (HDL). In a study of fasting plasma from twelve normolipemic subjects, the concentrations of both the apolipoprotein and lipid constituents were variable in the two density fractions, HDL_{2b} (d 1.063-1.100) and HDL_{2a} (d 1.100-1.130), and essentially constant in HDL_3 (d 1.130-1.210). Apolipoproteins A-I and A-II are major protein constituents of plasma HDL. Their daily transport into plasma from thoracic duct lymph was found to exceed their predicted synthesis. Thus, lymph HDL, which contains approximately 75% of lymph apoA-I and A-II, may to some extent represent recirculating HDL from the plasma. In a comparison of HDL cholesterol levels in cord blood from neonates from Cincinnati, OH and Leningrad, USSR, significant female-male differences existed in both populations while no significant Cincinnati-Leningrad differences were observed for either sex.

978

PHS-6040
(Rev. 10-76)

Project Description

Objective:

1) Characterization of the plasma concentrations of the apolipoprotein and lipid components of the human plasma high density lipoproteins (HDL) as they relate to three HDL subfractions, HDL_{2b}, HDL_{2a}, and HDL_3. This work was carried out in collaboration with Dr. E. N. Gerasimova, Myasnikov Institute, Moscow, USSR, and Dr. A. V. Nichols, Donner Laboratory, University of California, Berkeley, CA.

Methods Employed:

HDL (d 1.063-1.21 g/ml) from normolipemic individuals represents an admixture of at least three components: HDL_{2b} (d 1.063-1.100), HDL_{2a} (d 1.100-1.13), and HDL_3 (d 1.13-1.21) (D. Anderson et al, Atherosclerosis 29: 161-179, 1978). A simple two-step preparative method was developed to isolate each component by differential density ultracentrifugation. This method was applied to four aliquots of plasma from six normolipemic males and six normolipemic females to obtain the HDL_{2b}, HDL_{2a}, HDL_3, and total HDL fractions. Comparison of the particle size range corresponding to each HDL density fraction with that of native plasma HDL was carried out by porosity gradient electrophoresis in polyacrylamide gel (D. Anderson et al, Biochim. Biophys. Acta 493: 55-68, 1977). The concentrations of apolipoproteins (apo) A-I, A-II, and B in each fraction was determined by radial immunoassay and electroimmunoassay. The cholesteryl ester and unesterified cholesterol content of all fractions was determined with the Beckman oxygen electrode-enzymic analyzer (T. J. Bronzert and H. B. Brewer, Jr. Clin. Chem. 23: 2089-2098).

Major Findings:

For the 12 subjects studied, the mean plasma concentrations of the HDL lipid and apolipoproteins measured in the total HDL density fraction were statistically indistinguishable from the sum of the mean plasma concentrations of HDL_{2b}, HDL_{2a}, and HDL_3 lipid and apolipoprotein constituents. This simplified subfractionation procedure therefore does not effect significant redistribution of HDL lipid and apolipoprotein constituents among the three components during their separation. In specific, the following results were obtained: a) The apoA-I, apoA-II, unesterified cholesterol, cholesteryl ester, and phospholipid concentrations in both the HDL_{2b} and HDL_{2a} subfractions were directly related to their total HDL concentrations ($p<0.05$ for all cases). b) ApoB was detected only in the HDL_{2b} fraction. c) The concentrations of all lipid and apolipoprotein constituents in HDL_3 were not significantly related to their respective total HDL concentrations. d) HDL_2 cholesterol is indicated from these data to be the variable component of total HDL cholesterol among normolipemic individuals. It is not clear, however, that determination of HDL_2 cholesterol levels in epidemiological studies would establish this parameter as a better predictor of relative risk of atherosclerosis than total HDL cholesterol.

Objective:

2) Measurement of: i) the daily flow of apoA-I and apoA-II from thoracic duct lymph; ii) the distribution of these apolipoproteins among lymph lipoprotein classes; iii) the particle distribution and overall composition of lymph HDL. This work was carried out in collaboration with Dr. Frank T. Lindgren and Dr. Trudy Forte, Donner Laboratory, University of California, Berkeley, CA, and Dr. Gary Niblack, Vanderbilt University Medical Center, Nashville, TN.

Methods Employed:

Lymph was obtained by cannulation of the thoracic duct in two subjects undergoing lymph drainage in preparation for kidney transplantation. Lymph from a third subject who was non-uremic one week following successful kidney transplantation was obtained in the same manner. Lipid and apolipoprotein measurements were performed as in Objective 1). Analytical ultracentrifugation and gel permeation chromatography were employed to characterize the particle distributions of lymph lipoproteins. Electron microscopy of lymph lipoproteins, isolated in the presence or absence of an inhibitor (5,5'-dinitrobenzoic acid) of lecithin-cholesterol acyltransferase (LCAT) were performed.

Major Findings:

Measurement of all three parameters stated in Objective 2) yielded similar results for all three subjects. In specific, it was found that: a) No discoidal lipoprotein structures were detectable by electron microscopy in either whole lymph obtained in the presence or absence of LCAT inhibitor or any lymph lipoprotein fraction. b) Daily lymph transport into plasma of both apolipoproteins was 40-70% greater than their predicted plasma synthesis rates. This result constitutes evidence for recirculation of apoA-I and apoA-II between plasma and thoracic duct lymph. c) HDL isolated by preparative ultracentrifugation, heparin-Mn^{2+} precipitation, or Sepharose 6B-CL chromatography contained 71-84% of total lymph apoA-I and 77-89% of total lymph apoA-II. The remaining apoA-I and apoA-II was mostly in the lymph chylomicron fraction. d) The lymph HDL were larger (Stokes diameter: 9.2-12.7 nm) and exhibited faster peak flotation rates (peak $F^{o}_{1.20}$ 3.5-5.0) than plasma HDL from the same subject (Stokes diameter: 8.5-11.5 nm, peak $F^{o}_{1.20}$ 2.2-2.9). e) The triglyceride/total cholesterol ratio of lymph HDL was 5-7 times that of plasma HDL for each subject.

Objective:

3) Comparison of HDL cholesterol concentrations in the cord blood of male and female neonates in both Cincinnati, USA, and Leningrad, USSR, was undertaken in collaboration with both Prof. A. N. Klimov, Institute of Experimental Medicine, Leningrad, USSR, and Dr. C. J. Glueck, General Clinical Research, Cincinnati, OH.

Methods Employed:

The HDL cholesterol concentrations in cord blood plasma from 225 Cincinnati neonates and 200 Leningrad neonates were measured according to the heparin-Mn^{2+} precipitation technique of the Lipid Research Clinics (W. T. Friedewald et al. Clin. Chem. 19: 499-505).

Major Findings:

In the comparison of 225 Cincinnati and Leningrad male neonates and 200 female neonates there were either no significant differences in HDL cholesterol concentrations or the values were marginally higher in Cincinnati males. However, within both population groups, significant male-female differences were observed. In specific, median Cincinnati male neonate HDL cholesterol values were 29 mg/dl and female neonate values: 33 mg/dl whereas the median Leningrad male neonates were 28 mg/dl and female neonate values: 30 mg/dl.

Significance to Biomedical Research and the Program of the Institute:

The isolation, characterization, and quantitation of the lipoprotein particle and apolipoprotein distributions in normal individuals and patients with dyslipoproteinemia is vital to our knowledge of the transport and metabolism of lipids and plasma lipoproteins. Other body fluids (e.g., thoracic duct lymph) may also play a significant role in the homeostasis of normal lipid and lipoprotein metabolic pathways. The elucidation of these pathways in plasma and extraplasma fluids will facilitate our concept of lipid transport and catabolism in individuals with disorders of lipid metabolism and atherosclerosis.

Proposed Course:

The characterization of composition, particle distribution, and apolipoprotein content of plasma lipoproteins of normal subjects and patients with disorders of lipid metabolism will be compared to that of the lymphatic fluids in subjects under investigation. The transport of both lymph lipid and lymph apolipoprotein constituents into plasma will be characterized and related to human lipoprotein synthesis and catabolism.

Publications:

1. Anderson, D. W., Nichols, A. V., and Brewer, H. B., Jr.: Ultracentrifugal characterization of the human plasma high density lipoprotein distribution. Proceedings of the High Density Lipoprotein Methodology Workshop. DHEW Publication (in press).

2. Anderson, D. W., Bronzert, T. J., Schaefer, E. J., Niblack, G. D., Zech, L., Lindgren, F. T., Forte, T., and Brewer, H. B., Jr.: Transport of apolipoproteins A-I and A-II from human thoracic duct lymph into plasma. J. Lipid Res. (in press).

3. Schaefer, E. J., Anderson, D. W., Brewer, H. B., Jr., Levy, R. I., Danner, R. N., and Blackwelder, W. C.: Plasma-triglycerides in regulation of H.D.L.-cholesterol levels. The Lancet 1: 391, 1978.

4. Klimov, A. N., Glueck, C. J., Gartside, P. S., Magracheva, E. J., Livtchak, M. J., Shestov, D. B., Anderson, D. W., Tsang, R. C., Stein, E. A., and Steiner, D. M.: Cord blood high density lipoproteins - Leningrad and Cincinnati. Ped. Res. 13: 208, 1979.

5. Perova, N. V., Gerasimova, E. N., Polesski, V. A., Xhaltaev, N. G., and Anderson, D. W.: Determination of cholesterol concentrations in high density lipoprotein subfractions. Cardiologia (Russian) (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02016-02 MDB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Role of Acid Cholesterol Esterase in the Pathogenesis of Atherosclerosis

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

PI:	Robert Stark, M.D.	Clinical Associate	MDB	NHLBI
Other:	H. Bryan Brewer, Jr., M.D.	Chief	MDB	NHLBI
	Steve Demosky, B.A.	Chemist	MDB	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Molecular Disease Branch

SECTION
Peptide Chemistry

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The role of acid cholesterol esterase (ACE) in the metabolism of intracellular cholesetrol ester was evaluated in patients with premature cardiovascular disease. Acid cholesterol esterase activity, assayed in the lymphocytes of 36 patients, aged 25-45, with angiographically documented coronary artery disease, was significantly lower than that measured in 35 patients of similar age referred for evaluation of non-atherosclerotic heart disease. Multivariant analysis showed the reduction in ACE in the coronary artery group to be significant and independent of the incidence of any other recognized risk factor. Deficiency in cellular ACE, by limiting hydrolysis of intracellular cholesterol ester, may act as an independent risk factor in the atherogenic process.

PHS-6040
(Rev. 10-76)

Project Description

Objective:

cellular cholesterol ester was evaluated in patients with premature coronary artery disease. Acid cholesterol esterase is a lysosomal enzyme with acid pH optimum which is responsible for the hydrolysis of intracellular cholesterol ester and triglyceride.

Methods Employed:

Acid cholesterol esterase was determined in peripheral lymphocytes isolated by Ficoll-Paque utilizing the method of Böyum. The lymphocyte pellet was subjected to repeated freeze-thaw, and centrifuged to remove nuclei and cell fragments. Acid cholesterol esterase activity was determined using a fluorometric assay which employed 4-methylumbelliferyl oleate, a fluorgenic analog of cholesterol oleate. β-glucuronidase, a lysosomal marker enzyme, was assayed to evaluate the overall function of lysosomal enzymes.

The patients evaluated for premature cardiovascular disease and acid cholesterol esterase activity had anginal symptoms, or a myocardial infarction documented by EKG or enzymes, and had angiographically confirmed narrowing (>70%) of one or more coronary arteries. In addition, patients had a complete evaluation of established risk factors including lipoprotein phenotyping. Patients with known hyperlipidemia were excluded. Control patients were taken from patients undergoing evaluation of rheumatic or congenital heart disease with no symptoms of angina or had no significant lesions on coronary angiography.

Major Findings:

Seventy-one patients, aged 25 to 45, were studied. Thirty-six patients had premature coronary artery disease (CAD). Mean age in the CAD group was 39.6 (ranged 25 to 45), and there were 31 males and 5 females. Mean age of the control group was 36.2 (range 25 to 45), and there were 27 males and 8 females. There were no significant differences between the two groups in mean plasma cholesterol, triglycerides, body weight index, or incidence of hypertension, glucose intolerance, or positive family history. Tobacco use, however, averaged 27.9 pack years in the CAD group vs. 15.2 pack years in the controls ($p<.003$). There was no significant differences between the two groups in plasma VLDL or LDL cholesterol. HDL cholesterol, however, was significantly lower in the CAD patients than in controls (35.8 vs. 44.4 mg/dl; $p<.001$).

Purified lymphocyte preparations from the CAD group showed significantly lower ACE activity than those from the control group. Mean activity in the CAD patients was 38.9 nmol/min/mg compared to 55.9 nmol/min/mg in the controls ($p<.003$). Multivariant analysis showed the reduction in ACE activity to be significant ($p<.005$), and independent of the other two risk factors, HDL and

smoking.

Deficiency in cellular ACE, by limiting hydrolysis of intracellular cholesterol ester may act as an independent risk factor in the atherogenic process.

Significance to Biomedical Research and the Program of the Institute

Nearly fifty percent of patients with premature coronary artery disease have no currently recognized risk factor. The role of intracellular enzymes in normal cholesterol homeostasis, and disorders of these enzymes in patients with premature cardiovascular disease are as yet poorly understood. The finding that patients with premature cardiovascular disease have reduced levels of this lysosomal enzyme may be indicative of a new independent risk factor for the development of atherosclerosis.

Proposed Course:

The level of acid cholesterol esterase in monocytes separated by eltriation will be analyzed. In addition, fibroblasts obtained from these patients are currently being cultured and will be studied as to cholesterol content, and enzymic levels of acid cholesterol esterase.

Publications:

1. Stark, R. M., and Brewer, H. B., Jr.: Reduced acid cholesterol esterase activity in premature coronary artery disease. New Engl. J. Med. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02017-02 MDB

PERIOD COVERED
October 1, 1978 through September 30, 1979

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.	Staff Associate	MDB	NHLBI
Other:	Ernst J. Schaefer, M.D.	Staff Associate	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., Dan Steinberg, M.D., Ph.D., University of California, La Jolla, CA; Barbara Howard, Ph.D., Phoenix Clinical Research, Center Section, NIAMDI

LAB/BRANCH
Molecular Disease Branch

SECTION
Peptide Chemistry

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

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)

The plasma decay of radiolabeled apolipoprotein A-I and apolipoprotein A-II was analyzed in 14 normal individuals. The residence time of A-II was greater than A-I ($p<0.005$) indicating that A-I decays faster than A-II. Compartmental models were constructed separately for A-I which contained two plasma compartments, one decaying faster than the other. However, only a single plasma compartment was required for the A-II compartmental model. The extra plasma compartment in A-I was found to account for the decrease in A-I residence time. Triglyceride kinetics were studied in 59 normal or hypertriglyceridemic men utilizing radiolabeled glycerol. Analysis by a compartmental model revealed two pathways for the synthesis of VLDL-TG from glycerol. Differences in VLDL-TG kinetics found using ^{14}C-glycerol and ^{3}H-glycerol were related to differences in the kinetics of ^{14}C glycerol as compared to ^{3}H glycerol in only one of the synthesis pathways for VLDL-TG. Triglyceride kinetics were determined in male, obese, nondiabetic Pima Indians and were compared to normal weight, or obese nondiabetic male caucasian controls. By compartmental analysis, the FCR's for VLDL-TG were as follows: .42 hr obese Indians; .21 hr normal weight caucasians; and .32 hr for obese caucasian.

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PHS-6040
(Rev. 10-76)

Project Description

1) Objective:

Development of compartmental models for analysis of apoA-I and apoA-II metabolism in normal subjects and patients with disorders of lipoprotein metabolism and atherosclerosis.

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 at the National Bureau of Standards and the Periferial Data Processor-10 at the National Institutes of Health. These simulated results are compared to the experimental results and the connectivity (number of compartments) as well as flow of the model changed until a working model is developed. Using this model the volume of distribution of the apolipoproteins is estimated and compared to independent estimates of these volumes. After development of the compartmental model, the parameters of the model are adjusted using nonlinear least techniques squares techniques resulting in a minimal least square error. These have now been extended to studies in which differences in multicompartmental models developed using turnover data from two radiolabels on two different apolipoproteins in normal individuals have been compared.

Major Findings:

1. Residence times were determined for apoA-I and apoA-II in 14 normals by fitting simultaneaous plasma decay curves to the sum of exponentials. Detailed analysis indicated that the decay of apoA-I was faster than apo-II.

2. Using plasma and urine data, a model was constructed for apolipoprotein A-I. Using simulator plasma and urine data, a separate model was constructed for apoA-II metabolism. These two models were then analyzed and made similar in as many aspects as possible. An extra fast decaying plasma compartment was required for the A-I model which was not necessary for the apoA-II model. In addition, decay from a non-plasma compartment was required for the apoA-II model but not for the apoA-I model.

3. Data previously published in the literature was reanalyzed and compared to the above findings. All previously published data was found to be consistent with the new findings lending strength to the finding that the apoA-I was metabolized faster than apoA-II.

2) Objective:

1. Development of a multicompartmental model for triglyceride metabolism and the precursors of triglyceride metabolism.

2. Estimation of the parameters describing triglyceride metabolism including synthesis rate, residence time, and fractional catabolic rate of several well-defined groups of subjects.

Methods Employed:

Radiolabeled glycerol was administered to normal subjects and patients with varying degrees of obesity, diabetes, hypertriglyceridemia, and liver disease following analysis of the radiolabled glycerol data from the entire group of subjects. Previous multicompartmental models for very low density plasma lipoprotein were further developed and expanded. This new model was used to calculate the kinetic parameters of VLDL-triglyceride metabolism of all subjects studied. In addition, differences in metabolism associated with ^{3}H-glycerol and ^{14}C glycerol precursors were examined. The sensitivities of model parameters to changes in VLDL-TG kinetics and plasma glycerol precursor were also examined. When any group was large enough or extensively defined, estimates were made from the values and the dispersion of the values of the kinetic parameters for that subpopulation.

Major Findings:

1) Analysis of the kinetics of very low density lipoprotein triglyceride kinetics were performed in collaboration with Dr. Mones Berman (Laboratory of Theoretical Biology, NCI), Dr. Scott Grundy and Dr. Dan Steinberg (Veterans Administration Hospital and University of California, La Jolla, CA). Comparison of normal, mildly obese, and markedly obese subjects have shown that in general the markedly obese have increased synthesis rates for very low density lipoprotein triglycerides. In addition, obese subjects which are normal lipemic tend to have increased fractional catabolic rates when compared to normal subjects. Obese subjects with hypertriglyceridemia however tend to have normal catabolic rates of very low density lipoprotein triglyceride.

2) The tail of the kinetic curve for VLDL-TG was lower with the ^{3}H-glycerol precursor than in experiments employing the ^{14}C glycerol presursor however there was little difference in the peak values. Using the compartmental model it was shown that this difference is due to changes in one of two synthesis pathways for the conversion of labeled glycerol to very low density lipoprotein triglyceride.

3) In addition, using the compartmental model it was shown that very low density lipoprotein triglyceride kinetics were very sensitive to the fraction of very low density lipoproteins which beta have mobility when compared to the normal pre-beta mobility of the major fractions of very low

density lipoproteins. Very low density lipoprotein triglyceride metabolism was also very sensitive to the amplitude and slope of the tail of the very low density lipoprotein triglyceride activity curve. VLDL-TG metabolism was not sensitive to changes in glycerol metabolism however.

3) Objective:

Analysis of the very low density lipoprotein triglyceride kinetics in Pima Indians.

Methods Employed:

The Pima Indian nation of Native Americans are an interesting, well-defined group of subjects. Studies of this group were performed in collaboration with Dr. Barbara Howard and Dr. Peter Bennett (Phoenix Experimental Station, NIAMDD). These individuals are of interest because they have an increased incidence of diabetes, decreased incidence of lithogenic bile and cholesterol gallstones, and increased incidence of obesity greater than 150% of ideal body weight. In addition, they also represent a well-defined group in which insulin, glucose, cholesterol, and low density lipoprotein triglyceride kinetics have been previously studied. The very low density lipoprotein triglyceride kinetics in 15 normal triglyceridemic, non-diabetic Pima Indians has been examined using a multicompartmental model developed for triglyceride synthesis and metabolism. The connectivity (number of compartments as well as flow) of the model developed for caucasians was found to be adequate. The kinetic parameters were compared to two groups, normal weight (<125% ideal body weight) and obese (>160% ideal body weight) normal triglyceridemic caucasians. The fractional catabolic rate for very low density lipoprotein triglyceride for Pima Indians was increased (.42 h^{-1}) compared to normal (.21 h^{-1}) and obese (.32 h^{-1}) caucasians. The synthesis rate in Pima Indians (803 mg/hr) was equal to normal (727 mg/hr) but less than obese caucasians (1414 mg/hr).

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 model building. This type of theoretical analysis provides a uniform, systematic method for the comparison of experimental data and 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 apolipoprotein by drugs, diet, and genetic disease is also of significance since changes in these modulators 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 of longer duration. The overall objective will be the development of a comprehensive model of human lipoprotein metabolism by the incorporation of this information into the previously proposed LpB model. The formulation of an overall conceptualization of lipoprotein metabolism in normal individuals and in subjects with disorders 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 be the determination of which parameters remain invariant and variant when the systems are modulated by diet, drug, and transformed by genetic disease.

Publications:

1. Zech, L. A., Grundy, S. M., Steinberg, D., and Berman, M.: Kinetic model for production and metabolism of very low density lipoprotein triglyceride. J. Clin. Invest. 63: 129

2. Grundy, S. M., Moh, H. Y. I., Zech, L. A., Steinberg, D.: Transport of very low density lipoprotein triglycerides in varying degrees of obesity and hypertriglyceridemia. J. Clin. Invest. 63: 1274-1283.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02018-01 MDB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Immunolocalization of Apolipoproteins in Human Tissue

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

PI:	David E. Schwartz, M.D.	Clinical Associate	MDB	NHLBI
Other:	H. Bryan Brewer, Jr., M.D.	Chief	MDB	NHLBI
	Ernst J. Schaefer, M.D.	Staff Associate	MDB	NHLBI

COOPERATING UNITS (if any) Renu Virmani, M.D., Armed Forces Institute of Pathology; Denis M. McCarthy, M.D., DDB, NIAMDD; Robert Farnham, M.D., Pathology Department, Duke University, Durham, NC

LAB/BRANCH
Molecular Disease Branch

SECTION
Peptide Chemistry

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	

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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 application of peroxidase immunocytochemical techniques to human tissue utilizing antibodies to apolipoproteins (developed in our branch), was successfully demonstrated in our laboratory during the past year. Human jejunal tissue was analyzed for the presence of apolipoproteins A-I, A-II, and B in the epithelial cell biopsies from subjects, and patients with familial HDL deficiency (Tangier Disease) and abetalipoproteinemia. Apolipoproteins A-I and A-II were present in normal tissues and in intestinal samples from Tangier Disease. These studies are consistent with the view that both apoA-I and apoA-II are synthesized in patients with Tangier Disease. ApoA-I and apoA-II were also present in the intestinal cells of patients with abetalipoproteinemia.

These studies have been of major importance in our analysis of the sites of apolipoprotein synthesis in normals and has provided insight and raised interesting questions about apolipoprotein metabolism in dyslipoproteinemic subjects. Recently, steps have been undertaken to improve the technique to expand the tissues being investigated to include coronary arteries, liver, and lymphoreticular tissue.

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Project Description

Objective:

To identify the presence or absence of various apolipoproteins and their pattern of distribution in intestinal tissue, liver, and coronary arteries in normal and dyslipoproteinemic subjects.

Methods Employed:

A modification of Sternberger's peroxidase anti-peroxidase immunohistochemical technique (J. Histochem. Cytochem. 1970. 18: 315-333) utilizing unlabeled monospecific apolipoprotein antibodies was employed to link horseradish peroxidase to a specific tissue apolipoprotein. A chromagen is then precipitated at this site via a redox reaction involving hydrogen peroxide as the oxidizing agent.

Tissue samples are obtained in paraffin blocks from previous surgical biopsies and fresh tissue from autopsy specimens. These tissue samples are sliced on a cytrostat or microtome and transferred to gelantinized slides for peroxidase staining as well as red O, movat, and hexatoxylin/eosin staining when appropriate.

Major Findings:

1. ApoA-I, apoA-II, apoB are present in jejunal epithelial cells of fasting normal subjects.

2. ApoA-I, apoA-II, apoB are present in jejunal tissue of patients with Tangier disease. These studies are consistent with the view that the defect in Tangier disease is due to increased catabolism rather than defective synthesis of apoA-I and apoA-II.

3. ApoA-I, apoA-II, apoB, apoC-I, and apoC-III are present in atherosclerotic plaques in foam cells, in the cytoplasm of intimal and medial smooth muscle cells, and at sites of neutral lipid deposition.

Significance to Biomedical Research and the Program of the Institute:

LDL and HDL have been shown to be correlated with the incidence of coronary heart disease. Sites of synthesis of their constituent apolipoproteins provide insight into their metabolism. Furthermore, identification of apolipoproteins in coronary plaques may help unravel the etiology of such lesions.

Proposed Course:

Future research will be directed toward: 1) the completion of studies involving the localization of apolipoprotein in various tissues in subjects with various types of dyslipoproteinemia; 2) the analysis of new antibodies

prepared against several new apolipoproteins; and 3) the comparison of the peroxidase technique with immunofluorescent antibody techniques.

Publications:

1. Schwartz, D. E., Liotta, L., Schaefer, E. J., Brewer, H. B., Jr.: Localization of apolipoproteins A-I, A-II, and B in normal and Tangier intestinal mucosa. Metabolism (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02019-01

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Metabolism of Human Plasma Apolipoproteins

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

PI:	Richard E. Gregg, M.D.	Clinical Associate	MDB	NHLBI
Other:	Ernst J. Schaefer, M.D.	Staff Associate	MDB	NHLBI
	Loren A. Zech, M.D.	Staff Associate	MDB	NHLBI
	Leslie L. Jenkins, M.S.	Biologist	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, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.2	1.2	1.0

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The metabolism of C apolipoproteins has been studied in normal subjects. The metabolism of the C apolipoproteins are of major importance since apoC-II is the cofactor for lipoprotein lipase, the enzyme involved in the catabolism of triglyceride rich lipoproteins (chylomicrons and VLDL).

ApoC-II and apoC-III turnover studies were performed in 7 normal volunteers. VLDL, apoC-II, and apoC-III were radioiodinated, injected into the subjects and the decay of apoC-II and apoC-III from plasma and the plasma lipoprotein subclasses was followed with time to determine the parameters of their metabolism. The fractional catabolic rate of apoC-II was slightly shorter than apoC-III. The shape of the apoC-II and apoC-III decay curves were markedly different in VLDL when compared to HDL. The findings have been interrupted to indicate that apoC-II and apoC-III are metabolized independently and that the metabolism of these apolipoproteins is different in separate lipoprotein density classes.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

These studies were performed to determine the kinetic parameters of apoC-II and apoC-III metabolism in normal individuals. The metabolism of apoC-II is of major importance since it is a cofactor for the enzyme lipoprotein lipase, the enzyme involved in the metabolism of triglyceride rich lipoproteins, chylomicrons, and very low density lipoproteins. With this data we can gain further insights into the factors regulating the metabolism of these apolipoproteins, help determine how these apolipoproteins control lipid metabolism, and compare the metabolism of apoC-II and apoC-III in normal individuals with patients with dyslipoproteinemia.

Methods Employed:

The normal subjects were placed on isocaloric balanced diets with a low cholesterol level and high P/S ratio. The VLDL, apoC-II, and apoC-III were radioiodinated by the method of McFarlane as outlined in previous annual reports. These radiolabeled apolipoproteins or lipoproteins were injected into the subjects and timed blood samples were obtained. The lipoprotein subclasses were separated by density centrifugation and the apolipoproteins isolated by TMU precipitation and polyacrylamide gel electrophoresis. The radioactivity was determined in a standard gamma counter.

Major Findings:

ApoC-II and apoC-III have plasma residence times of approximately 1 day with the apoC-III residence time being slightly but significantly shorter than the residence time of apoC-II. The shape of the apoC-II and apoC-III decay curves were markedly different in VLDL when compared to HDL. The findings suggest that apoC-II and apoC-III are metabolized independently and that they are metabolized differently in separate lipoprotein density classes.

Significance to Biomedical Research and the Program of the Institute:

Elevated triglyceride and VLDL levels are associated with a number of different medical diseases. ApoC-II and apoC-III are major lipoproteins of VLDL and apoC-II activates lipoprotein lipase. These studies will give further insight into factors regulating the metabolism of triglyceride and VLDL in normal subjects, and patients with disorders of triglyceride metabolism and hyperlipidemia.

Proposed Course:

It is planned to continue to study the metabolism of apoC-II and apoC-III in normal subjects and to study their metabolism in patients with dyslipoproteinemias. In adddition, it is planned to begin similar studies of apoE metabolism.

Publications:

None

Annual Report of the Pathology Branch
Division of Intramural Research
National Heart, Lung, and Blood Institute
October 1, 1978 through September 30, 1979

Investigations during the above time period as in previous years have centered primarily on studies of coronary, congenital, valvular, and myocardial heart diseases.

CORONARY HEART DISEASE

During the past 2 years, a number of studies have been done on the major coronary arteries in patients with fatal coronary heart disease. These studies have centered around what we have called "quantitation" of the degrees of coronary arterial narrowing in each of the 4 major (right, left main, left anterior descending, and left circumflex) coronary arteries. Since the right coronary artery in adults is approximately 10 cm in length, the left anterior descending about 10, the left circumflex about 6, and the left main 1 cm, a total of about 28 cm of major coronary artery have been studied in each patient. Each 1 cm segment of coronary artery has been subdivided into two 0.5 cm segments, and a histologic section stained by the Movat method has been examined in each 5 mm segment. The degrees of cross-sectional area narrowing in each 5-mm segment has been grouped into 4 categories: 0-25%, 26-50%, 51-75%, and 76-100%. During the past 1 year, several studies have been completed. The major purpose has been to describe the extent of the coronary narrowing in patients with fatal coronary heart disease, and secondly, to learn if differences in degrees of narrowing occurred in patients with the various subsets of coronary heart disease.

The first study concerned 31 necropsy patients with sudden coronary death. A total of 1,564 five-mm segments were examined and the findings were compared to those in 25 control subjects of similar age and sex. A total of 1105 mm segments of major coronary artery were studied in the control subjects. Of the 1,564 five-mm segments in the 31 study patients, 556 (36%) were 76 to 100% narrowed in cross-sectional area by atherosclerotic plaque (controls = 3%); 536 (34%) were 51 to 75% narrowed (controls = 23%); 360 (23%) were 26 to 50% (controls = 42%), and 111 segments (7%) were 0 to 25% narrowed. The amount of severe (> 75%) narrowing of the right, left anterior descending and left circumflex coronary arteries were similar. The amount of severe narrowing in the distal one-half of these 3 arteries was similar to that in the proximal halves of these arteries.

The next study concerned quantitation in 22 patients with unstable angina pectoris, and the observations in them were compared to those in 20 control subjects. Of 1049 mm segments of the 4 major coronary arteries examined in the 22 patients, 497 (47%) were 76 to 100% narrowed in cross-sectional area by atherosclerotic plaques (controls = 1%); 304 (29%) were 51 to 75% narrowed (controls = 9%); 454 (48%) were 26 to 50% narrowed, and 119 (11%) were 0 to 25% narrowed (controls= 22%). The amount of severe narrowing of the right, left anterior descending and left circumflex coronary arteries by

atherosclerotic plaques was similar. The amount of severe narrowing in the distal one-half of the right and left anterior descending coronary arteries, however, was significantly less than in the proximal halves of these 2 arteries.

The next study concerned 27 necropsy patients with fatal acute transmural myocardial infarction and the findings were compared to those in 22 control patients. Of 1430 mm segments examined in the 27 patients, 484 (34%) were 76 to 100% narrowed in cross-sectional area by atherosclerotic plaques (controls = 3%); 528 (38%) were 51 to 75% narrowed (controls = 25%); 319 (23%) were 26 to 50% (controls = 44%) and 72 segments (5%) were 0 to 25% narrowed (controls = 28%). The amount of severe narrowing of the right, left anterior descending and left circumflex coronary arteries by atherosclerotic plaques was similar. The amount of severe narrowing in the distal one-half of the right, left, anterior descending and left circumflex coronary arteries was similar to that in the proximal halves of these arteries.

The next study concerned 18 necropsy patients with healed myocardial infarcts and death from chronic congestive heart failure. Not only were the degrees of coronary narrowing in them quantitated, but the size of the myocardial infarcts also was quantitated. Each of the 18 patients had very dilated right and left ventricular cavities, hearts weighing more than 450 gms., intractable congestive failure for longer than 3 months, and half had intraventricular mural thrombi. Of 1012 five-mm segments of the 4 major coronary arteries examined, 298 (29%) were 76 to 100% narrowed in cross-sectional area by atherosclerotic plaques (in 16 control subjects = 6%); 370 (37%) were 51 to 75% narrowed (controls = 35%), 227 (23%) were 26 to 50% narrowed (controls = 43% and 117 (11%) were 0 to 25% narrowed (controls = 16%). The amount of severe narrowing of the right, left anterior descending and left circumflex coronary arteries was similar. The amount of severe narrowing in the distal one-half of the right, left anterior descending and left circumflex coronary arteries was similar to that in the proximal halves of these arteries. In all 18 patients the healed infarcts involved > 40% of the left ventricular wall.

The next group of patients were 18 with non-fatal healed transmural myocardial infarcts, and a fatal non-cardiac condition. After healing the myocardial infarcts none of these 18 patients ever had evidence of myocardial ischemia thereafter. Therefore, this group of 18 patients is diametrically opposite the group described in the paragraph above. The infarcts in these patients involved an average of 30% of the basal half of left ventricle and an average of 38% of its apical half. Of 924 five-mm segments examined, 292 (31%) were 76 to 100% narrowed in cross-sectional area by atherosclerotic plaques (controls = 5%); 319 (35%) were 51 to 75% narrowed (controls = 34%); 211 (23%) were 26 to 50% narrowed (controls = 44%), and 102 (11%) were 0 to 25% narrowed (controls = 17%). The amount of severe narrowing was similar in the left anterior descending and left circumflex coronary arteries but the right was more severely narrowed. The amount of severe narrowing in the proximal and distal halves of the right, left anterior descending and left circumflex coronary arteries was similar. Thus, these patients had the same amount of severe coronary narrowing as did the patients with healed myocardial

infarcts who were left with intractable congestive heart failure.

The above described studies are the first to describe in quantitative detail the degree and extent of luminal narrowing by atherosclerotic plaques in each of the 4 major epicardial coronary arteries in various subgroups of patients with coronary heart disease. Of over 100 patients studied, 36% of the major coronary tree was 76 to 100% narrowed in cross-sectional area by atherosclerotic plaque and another 36% was 51 to 75% narrowed for a total of 72% > 50% narrowed. Thus, coronary atherosclerosis among patients with fatal coronary heart disease is a diffuse process. The patients with unstable angina pectoris had the highest percent of five-mm segments 76 to 100% narrowed in cross-sectional area by atherosclerotic plaque and the patients with healed myocardial infarcts, irrespective of whether or not they died from progressive congestive heart failure or from a non-cardiac condition, had the least percent of five-mm segments 76 to 100% narrowed. The patients with acute myocardial infarction and those with sudden coronary death were intermediate and similar. Thus, the patients with the least amount of myocardial damage, i.e., angina pectoris, had the severest degree of coronary narrowing, and those with the severest degree of myocardial damage, namely healed myocardial infarts, had the least degree of coronary narrowing.

While these quantitative studies were being carried out, a study was also done to confirm that the technique utilized in these quantitative studies was a good one. The percent of luminal narrowing in the cross-sections of coronary artery was determined by visual inspection of Movat stained histologic sections magnified 20 to 50 times. An analysis was made of 559 five-mm segments from 61 coronary arteries to see what was the inter-observer error by visual inspection under microscopy. Three observers examined each of these 559 sections, and the agreement between the 3 observers was 95%. Thus, the technique was a good one. In addition, the accuracy of the coronary narrowing by visual inspection under microscopy was compared to an accepted prototype, namely video planimetry because this technique has a known accuracy of over 97%. Kappa analysis of the results of visual inspection under microscopy and video planimetry showed a strong overall agreement (average = 72%) for each of 3 observers. Thus, visual inspection under microscopy, the technique used in the above quantitative studies, not only has a strong inter-observer agreement, but it also is nearly identical to that obtained by video planimetry.

Although several sudies comparing the accuracy of coronary cineangiograph during life to the degree of narrowing observed at necropsy have been carried out in the past, none has utilized the quantitative technique described above. Consequently, we studied the coronary arteries quantitatively in 10 patients, a total of 61 coronary arteries, in patients who had coronary cineangiography during life and died within 30 days of the angiographic procedure. The angiograms had been read by 3 different angiographers, and their readings were compared to that observed histologically at necropsy. No overestimations of degrees of narrowing were made angiographically. Of 11 coronary arteries narrowed 0 to 50% in cross-sectional area histologically, none were underestimated angiographically; of 8 narrowed 51 to 75% histologically, 7 had been underestimated, and of 42 narrowed 76 to 100%

histologically, 17 (40%) were underestimated angiographically. The coronary atherosclerotic plaquing was diffuse in 90% of 467 five-mm segments of coronary artery examined, and this diffuseness of the atherosclerotic process appeared to be the major reason for angiographic underestimation of the coronary narrowing.

For years it has been believed that patients with diabetes mellitus have more coronary atherosclerosis than patients of similar age and sex without diabetes. We studied 229 necropsy patients with diabetes with onset after age 30 years, and 164 had clinical evidence of coronary heart disease, and 65 did not. The observations in the 229 diabetic patients were compared to those in 183 age, sex-matched non-diabetic patients with coronary heart disease. The average number of 3 major (right, left anterior descending, left circumflex) coronary arteries narrowed >75% in cross-sectional area by atherosclerotic plaque was similar in the diabetic patients with and without clinical evidence of coronary heart disease, and both groups were similar to the non-diabetic patients with coronary heart disease. The percent of each of the 3 major coronary arteries narrowed >75% in cross-sectional area by atherosclerotic plaque was similar in the 2 diabetic study groups and in the non-diabetic patients. Furthermore, the percent of proximal and distal halves of these arteries narrowed to this degree was similar. The duration of, the type of treatment for, and the level of fasting blood sugar in the diabetic patients appeared to have no significant effect on the amount of severe coronary narrowing observed.

For several decades it has been believed that bilateral oophrectomy in menstruating women accelerates coronary atherosclerosis. The reported studies which suggested the above to be a fact were analyzed in detail, and we concluded that at least in humans, pre-menopausal castration has not been conclusively demonstrated to accelerate coronary atherosclerosis.

For some time severe thrombocytosis has been believed to be responsible for arterial thrombosis on rare occasion. During this past year we studied a 22-year-old man at necropsy, and he had had thrombocytosis for nearly a year and arterial thrombi in several arteries, including the coronaries, and the result was acute myocardial infarction. The patient was the first to have thrombocytosis actually documented to be the cause of acute myocardial infarction. Other than the thrombi within his heart there was no underlying atherosclerosis, and no other predisposing factor for coronary thrombosis. A review of reported patients with vascular occlusion associated with thrombocytosis actually indicated that thrombi have infrequently been confirmed as the mechanism of the vascular occlusion. The patient we described clearly had vascular thrombosis as a consequence of thrombocytosis.

CONGENITAL HEART DISEASE

For many years there has been some puzzlement as to why some patients with pulmonic valve stenosis have a right-to-left shunt at the atrial level and others have a left-to-right shunt. To attempt to clarify the mechanism for the atrial shunting in patients with valvular pulmonic stenosis, 127 patients who underwent pulmonic valvulotomy for valvular pulmonic stenosis with intact ventricular septum and without obstruction to left ventricular inflow or outflow were reviewed. Of the 127 patients, 30 (24%) pre-operatively by dye-dilution curves had shunting at the atrial level: in 19 (63%) the

shunt was right-to-left, and in the other 11 (27%), entirely left-to-right. The patients with right-to-left interatrial shunts had severe pulmonic valve stenosis (average peak systolic pressure gradients of 120 ± 11 mm Hg) and small (average diameter = 1.1 cm) sized defects in the atrial septum. In contrast, the patients with left-to-right shunts had only mild to moderate pulmonic valve stenosis (average peak systolic pressure gradient = 60 ± 5 mm Hg) and relatively large (average diameter = 2.8 cm) defects in the atrial septum. The patients with right-to-left interatrial shunts had no significant differences in right versus left atrial pressures. The patients with left-to-right interatrial shunts, however, had left atrial pressures significantly greater than right atrial pressures. No significant differences were found in ventricular end-diastolic pressures.

VALVULAR HEART DISEASE

During the past 20 years, a number of prosthetic cardiac valves have been evaluated anatomically in this laboratory. The porcine valve has received particular attention in recent years. Light and electron microscopic studies were made in 30 porcine valvular heterografts removed from 28 patients either at necropsy or at reoperation. Early changes (less than 2 months) consisted of deposition of fibrin, macrophages and multinucleated giant cells on the vascular 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) were characterized by progressively severe damage to the collagen, formation of aggregates of platelets on the valvular surfaces, lipid deposition, erosion of the surfaces, and development of calcific deposits. These alterations in collagen appear to limit the long-term durability of the porcine valvular heterograft.

A number of studies from this laboratory have described observations in the heart in patients with prosthetic valve endocarditis. Relatively little information is available on infection involving porcine bioprostheses. As a consequence, gross, histologic and ultrastructural changes in 4 patients with bacterial infection involving porcine heterografts were described. Study of our 4 patients indicates that the infection develops in the fibrin layer that covers the cusps, that the infection can involve the collagen in the leaflets and is uncommonly, in contrast to infection involving the rigid frame prosthesis, associated with valve ring abscesses.

MYOCARDIAL HEART DISEASE

Last year several patients with hypertrophic cardiomyopathy and transmural myocardial infarction were described. This year we studied a patient with idiopathic dilated cardiomyopathy who also had transmural myocardial infarcts which had healed involving the right ventricular free wall, ventricular septum and left ventricular free wall, and both the intramural and epicardial coronary arteries were normal. Thus, not only can transmural infarction of uncertain etiology be a complication of hypertrophic cardiomyopathy, but it can also be a complication of idiopathic dilated cardiomyopathy.

PULMONARY DISEASE

From the Pulmonary Branch of the NHLBI, many lung biopsies are done each year. Both light and electronmicroscopic examinations of these biopsies are carried out. Of 19 patients with various fibrotic lung disorders, 9 were found to have nuclear inclusions in a small percentage of cuboidal alveolar epithelial cells. These inclusions consisted of masses of tubules and resembled nuclear tubules which occur in other cell types under conditions of rapid growth or specific hormonal stimulation. The significance of these occlusions is unclear.

In 17 patients with fibrotic lung disorders in whom the lung biopsy was studied by electron microscopy, extensive alterations in pulmonary mast cells were observed. The mast cells were increased in number and showed evidence of degranulation and of migration through the alveolar epithelial cell layer into the alveoli. The significance of these observations is unclear.

In recent years anchoring fibrils have been described in a number of human lung disorders from this laboratory. Study of normal canine respiratory tree disclosed these anchoring fibers also to be present. They were noted in the trachea, bronchi, bronchioles and tracheobroncheal glands, and they appear to reinforce the attachment of the epithelial basal lamina to the underlying connective tissue.

MISCELLANEOUS STUDIES

In conjunction with 2 investigators at the Center for Disease Control in Atlanta, Georgia, we studied 17 patients who had died suddenly and unexpectedly and who had been avid dieters using the liquid protein modified fast diet. Of the 17 patients, 16 were women, most were young (average age = 37 years), and most lost a massive amount of weight (average = 41 kilograms or 35% of their pre-diet weight) over a short period of time (average = 5 months). Eight had one or more episodes of syncope. Electrocardiograms in 10 patients disclosed that all had episodes of ventricular tachycardia and prolongation of the Q-T interval, and 9 had diminished amplitude of the QRS complexes (low voltage). Histologic study of the left ventricular myocardium in 14 patients disclosed attenuated myocardial fibers in 12, increased lipofuscin pigment in 11 and mononuclear cell myocarditis in one. Similar histologic findings, however, also were found in 16 cachetic control subjects studied in similar fashion, but electrocardiograms in them showed no prolongation of Q-T intervals or episodes of ventricular tachycardia. Thus, semi-starvation, particularly in the face of antecedent obesity, is a cause of acquired Q-T interval prolongation, and repeated electrocardiograms are recommended in patients on semi-starvation diets for treatment of obesity.

The intra-aortic balloon counterpulsation device was introduced several years ago, and although a few complications from it have been described, no systemic morphologic study in patients who had had this balloon assist device inserted have been carried out. We studied 45 patients who died after insertion of the balloon and 16 at necropsy (36%) were found to have one or more complications (total = 20) related to use of the device. The 20

complications consisted of dissection of the aorta and/or its distal branches (3), extremity ischemia (1), and local wound infection (1). Of the total 20 complications, only 4 were suspected before death. Although the operating team frequently encountered no difficulty at the time of insertion of the device, 12 of the 20 complications were a direct result of its insertion. Thus, clinical evaluation of complications related to use of the balloon assist device underestimates their frequency. Most complications are a consequence of insertion of the device, not consequences of its being in place.

During the past 20 years many patients with various diseases of the aorta have been studied at necropsy in this laboratory. A review of these patients was made. The generalized dilatation of the aorta which occurs with aging appears to be the result of degeneration of the elastic fibers in the aortic media. Why atherosclerosis appears to be more common in the abdominal aorta than in other portions of the aorta is still not fully explained. The exception to this are patients with type II hypolipoproteinemia, and these individuals tend to have more atherosclerosis in the ascending than in the abdominal aorta. Aneurysms of the abdominal aorta are nearly always fusiform in type and aneurysms located in the descending thoracic aorta are more commonly saccular in type. Saccular aneurysms of the descending thoracic aorta are relatively infrequent, however, in the absence of associated fusiform aneurysms of the abdominal aorta. Why abdominal aortic obstruction occurs in some patients rather than aneurysmal dilatation is unclear but examination of several patients suggested that the abdominal aorta calcifies early in the patients who later go on to obstruct and that the calcific wall prevents expansion. Considerable evidence was gained that aortic dissection was simply the result of systemic hypertension. Although there are nearly 25 million patients with systemic hypertension in the USA, dissection fortunately is infrequent, but dissection simply does not occur unless systemic hypertension had been present. Cardiovascular syphilis continues to be observed at necropsy. Cardiovascular syphilis is for the most part limited to the tubular portion of aorta and does not involve the portion of aorta behind the sinuses of Valsalva. In contrast, in ankylosing spondylitis the wall of aorta behind the sinuses of Valsalva is preferentially involved and the process tends to spare the tubular portion of ascending aorta.

During the 1950's, much was written on negative pressures in the right and left ventricles, but little was written on that subject in the 1960's or 1970's, and previous work had involved only non-human animals. A man was studied at necropsy who died following a gunshot wound which entered his right atrium and exited the right ventricle without entering the cardiac septum or the left side of the heart. At necropsy, the left atrial appendage was found to be inverted and invaginated into the mitral orifice. The invagination of the left atrial appendage was viewed as anatomic evidence that a negative left ventricular pressure was created as the left ventricular volume rapidly fell from right-sided cardiac exsanguination. The prerequisite for creation of a negative pressure in the ventricles during diastole is an extreme diminution in left ventricular volume, in our patient, from right sided cardiac bleeding. Explanations other than a vaccum effect

of the left ventricle during diastole cannot explain the invagination of their left atrial appendage in our patient.

Not infrequently, drugs have toxic actions on the heart. A vasodilating antihypertensive agent called minoxidil was found to produce myocardial lesions in beagle dogs. These lesions consisted of epicardial and endocardial hemorrhages, inflammatory cells in the walls of small coronary arteries and papillary muscle necrosis. The mechanism of the production of these lesions by this drug is unclear.

From study of myocardial tissue by electron microscopy in 169 patients with diverse conditions, tubulorecticular structures were observed in the myocardial cells in 5 or 3%. Their precise significance, however, was not determined.

During the past 15 years several innovations in technique of histology have been introduced by the personnel in the histology laboratory of the Pathology Branch. During the past year a dry-ice container mounted on a microtome for continuous cooling of tissue-containing paraffin blocks during sectioning was designed and built and is now utilized daily.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03136-01 PA

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

The Amount of Atherosclerotic Plaquing in the Coronary Arteries in Fatal Acute Transmural Myocardial Infarction: The Quantitative Approach

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

PI: William C. Roberts, Chief, Pathology Branch, NHLBI
Other: Ancil A. Jones, Clinical Associate, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A quantitative analysis of the degree and extent of coronary arterial narrowing by atherosclerotic plaques in the entire lengths of each of the four major coronary arteries in 27 necropsy patients with transmural acute myocardial infarction (AMI) is described at necropsy, and the observations in them are compared to those made in 22 control subjects. Of the 1403 five-mm segments examined in the 27 AMI patients (avg 52 per patient), 484 (34%) were 76-100% narrowed in cross-sectional area by atherosclerotic plaques (controls = 3%); 528 (38%) were 51-75% narrowed (controls = 25%); 319 (23%) were 26-50% (controls = 44%) and only 72 segments (5%) were < 25% narrowed (controls = 28%).

1005

Project Description: A quantitative analysis of the degree and extent of coronary arterial narrowing by atherosclerotic plaques in the entire lengths of each of the four major coronary arteries in 27 necropsy patients with transmural acute myocardial infarction (AMI) is described at necropsy, and the observations in them are compared to those made in 22 control subjects. Of the 1403 five-mm segments examined in the 27 AMI patients (avg 52 per patient), 484 (34%) were 76-100% narrowed in cross-sectional area by atherosclerotic plaques (controls = 3%); 528 (38%) were 51-75% narrowed (controls = 25%); 319 (23%) were 26-50% (controls = 44%) and only 72 segments (5%) were $\leq$ 25% narrowed (controls = 28%). The amount of severe (>75%) narrowing of the right, left anterior descending and left circumflex coronary arteries by atherosclerotic plaques was similar. Additionally, the amount of severe (> 75%) narrowing in the distal one-half of the right, left anterior descending and left circumflex coronary arteries was similar to that in the proximal halves of these three arteries.

Publications: Roberts, W.C., and Jones, A.A.: The amount of atherosclerotic plaquing in the coronary arteries in fatal acute transmural myocardial infarction: The quantitative approach. Proceedings of The International Meeting on Myocardial Infarction, Excerpta Medica, 1979 (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03137-01 PA

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Quantification of Coronary Arterial Narrowing at Necropsy in Fatal Acute Myocardial Ischemia

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

PI: William C. Roberts, Chief, Pathology Branch, NHLBI
Other: Ancil A. Jones, Clinical Associate, Pathology Branch, NHLBI
Renu Virmani, Staff Fellow, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, 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)

Each of the 4 major epicardial coronary arteries in 80 patients with fatal acute coronary events was divided into 5-mm long segments and a Movat-stained histologic section was prepared and examined from each segment. An average of 56 five-mm long segments were examined from each of the 80 patients. Of the 4016 five-mm segments of coronary artery examined, 1538 (38%) were 75 to 100% narrowed in cross-sectional area by atherosclerotic plaque (controls = 3%), another 34% of the segments were 51 to 75% narrowed (controls = 22%), 20% were 26 to 50% narrowed (controls = 44%) and only 8% were 0-25% narrowed (controls = 31%). Thus, coronary atherosclerosis among patients with fatal acute coronary events is diffuse and severe.

1007

PHS-6040
(Rev. 10-76)

Project Description: Each of the 4 major epicardial coronary arteries in 80 patients with fatal acute coronary events was divided into 5-mm long segments and a Movat-stained histologic section was prepared and examined from each segment. An average of 56 five-mm long segments were examined from each of the 80 patients. Of the 4016 five-mm segments of coronary artery examined, 1538 (38%) were 75 to 100% narrowed in cross-sectional area by atherosclerotic plaque (controls = 3%), another 34% of the segments were 51 to 75% narrowed (controls - 22%), 20% were 26 to 50% narrowed (controls = 44%) and only 8% were 0-25% narrowed (controls = 31%). Thus, coronary atherosclerosis among patients with fatal acute coronary events is diffuse and severe. The 22 patients with clinically unstable angina pectoris had the highest percent of 5-mm long segments 76 to 100% narrowed in cross-sectional area by atherosclerotic plaque. namely 48%; the 27 patients with acute myocardial infarction and the 31 patients with sudden cardiac death had a similar percent (about 35%) of coronary segments narrowed 76 to 100%. Among the 80 necropsy patients studied, those with the least amount of myocardial damage, i.e., angina pectoris, had the severest degree of coronary narrowing, and those with the severest degree of myocardial damage, i.e., acute myocardial infarction, had less severe coronary narrowing.

Publications: Roberts, W.C., Jones, A.A., and Virmani, R.: Quantification of coronary arterial narrowing at necropsy in fatal acute myocardial ischemia. Proceedings of International Symposium on Quantification of Myocardial Ischemia (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03139-01 PA

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Calcific Pulmonic Stenosis in Adulthood: Treatment by Valve Replacement (Porcine Xenograft) with Postoperative Hemodynamic Evaluation

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

PI: Edgar A. Covarrubias, Staff Cardiologist, DC General Hospital

Other: Mazhar U. Sheikh, Staff Cardiologist, DC General Hospital
Jeffrey M. Isner, Staff Associate, Pathology Branch, NHLBI
Mario Gomes, Staff Surgeon, Georgetown University School of Medicine
Charles A. Hufnagel, Chief of Cardiovascular Surgery, Georgetown University School of Medicine
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)
DC General Hospital, Washington, D.C., and Georgetown University School of Medicine, Washington, D.C.

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH 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 features are described in a 56-year-old man with isolated, calcific, severe, pulmonic valve stenosis. The calcific deposits were located on the ventricular aspects of the pulmonic valve, opposite to the location (arterial aspect) of calcific deposits on stenotic aortic valves, and calcific deposits also were present in the tricuspid-valve anulus.

1009

Project Description: Clinical and morphologic features are described in a 56-year-old man with isolated, calcific, severe, pulmonic valve stenosis. The calcific deposits were located on the ventricular aspects of the pulmonic valve, opposite to the location (arterial aspect) of calcific deposits on stenotic aortic valves, and calcific deposits also were present in the tricuspid valve anulus. The pulmonic valve was replaced with a porcine xenograft, but in view of the 36 mm Hg peak systolic pressure gradient across the prosthesis 3 months postoperatively, valve commissurotomy or partial valvulectomy may have been preferred.

Publications: Covarrubias, E.A., Sheikh, M.U., Isner, J.M., Gomes, M., Hufnagel, C.A., and Roberts, W.C.: Calcific pulmonic stenosis in adulthood: Treatment by valve replacement (porcine xenograft) with postoperative hemodynamic evaluation. Chest 75: 399-402, 1979

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03140-01 PA

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Quantitation of Coronary Arterial Narrowing at Necropsy in Sudden Coronary Death: An Analysis of 31 Patients and Comparison of Findings to Those in 25 Control Subjects

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

PI: William C. Roberts, Chief, Pathology Branch, NHLBI
Other: Ancil A. Jones, Clinical Associate, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A quantitative analysis of the degree and extent of coronary arterial narrowing by atherosclerotic plaques in the entire lengths of each of the 4 major coronary arteries in a group of patients dying suddenly from coronary heart disease ("sudden coronary death") is described at necropsy for the first time. Of the 1564 five-mm segments examined in the 31 study patients, 557 (36%) were 76-100% narrowed in cross-sectional area by atherosclerotic plaques (controls = 3%); 536 (34%) were 51-75% narrowed (controls = 22%) 360 (23%) were 26-50% (controls = 42%) and only 111 segments (7%) were < 25% narrowed (controls = 33%).

1011

Project Description: A quantitative analysis of the degree and extent of coronary arterial narrowing by atherosclerotic plaques in the entire lengths of each of the 4 major coronary arteries in a group of patients dying suddenly from coronary heart disease ("sudden coronary death") is described at necropsy for the first time. A total of 1564 five-mm long segments of the left main, left anterior descending, left circumflex and right coronary arteries were examined in 31 patients with sudden coronary death and the observations in them were compared to those made from examination of 1100 five-mm segments of major epicardial coronary artery in 25 control subjects. An average of 25 cm (50 five-mm segments) of coronary artery were examined from each patient and an average of 22 cm (44 five-mm segments) from each control subject. Of the 1564 five-mm segments examined in the 31 study patients, 557 (36%) were 76-100% narrowed in cross-sectional area by atherosclerotic plaques (controls = 3%); 536 (34%) were 51-75% narrowed (controls = 22%) 360 (23%) were 26-50% (controls = 42%) and only 111 segments (7%) were $\leq$ 25% narrowed (controls = 33%). The amount of severe (>75%) narrowing of the right, left anterior descending and left circumflex coronary arteries was similar; therefore, the concept that "the left anterior descending coronary artery is the artery of sudden death" should be discarded. Additionally, the amount of severe (>75%) narrowing in the distal one-half of the right, left anterior descending and left circumflex coronary arteries was similar to that in the proximal halves of these 3 arteries. The number of 5-mm coronary segments narrowed 76 to 100% in cross-sectional area in the 31 study patients was not affected by the patient's age at death, sex, presence or absence of previous angina pectoris or myocardialinfarction, or the weight of the heart.

Publications: Roberts, W.C., and Jones, A.A.: Quantitation of coronary arterial narrowing at necropsy in sudden coronary death: An analysis of 31 patients and comparison of findings to those in 25 control subjects. Amer J Cardiol 44: 39-45, 1979

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03141-01 PA

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
The Amount of Coronary Narrowing in the 4 Major Epicardial Coronary Arteries in Unstable Angina Pectoris: The Quantitative Approach

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

PI: William C. Roberts, Chief, Pathology Branch, NHLBI
Other: Renu Virmani, Staff Fellow, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A quantitative analysis of the degree and extent of coronary arterial narrowing by atherosclerotic plaques in the entire lengths of each of the 4 major epicardial coronary arteries in 22 patients with unstable angina pectoris is described at necropsy and the observations are compared to those in 20 control subjects. Of 1049 five-mm long segments of the left main, left anterior descending, left circumflex and right coronary arteries examined in the 22 patients (average 48 per patient), 497 (47%) were 76-100% narrowed in cross-sectional area by atherosclerotic plaques (controls = 1%); 304 (29%) were 51-75% narrowed (controls = 29%); 454 (48%) were 26-50% narrowed and only 119 (11%) segments were less than 26% narrowed (controls = 22%).

1013

Project Description: A quantitative analysis of the degree and extent of coronary arterial narrowing by atherosclerotic plaques in the entire lengths of each of the 4 major epicardial coronary arteries in 22 patients with unstable angina pectoris is described at necropsy and the observations are compared to those in 20 control subjects. Of 1049 five-mm long segments of the left main, left anterior descending, left circumflex and right coronary arteries examined in the 22 patients (average 48 per patient), 497 (47%) were 76-100% narrowed in cross-sectional area by atherosclerotic plaques (controls = 1%); 304 (29%) were 51-75% narrowed (controls = 29%); 454 (48%) were 26-50% narrowed and only 119 (11%) segments were less than 26% narrowed (controls = 22%). The amount of severe (>75%) narrowing of the right, left main, left anterior descending and left circumflex coronary arteries by atherosclerotic plaques was similar. The amount of severe (>75% narrowing in the distal one half of the right and left anterior descending coronary arteries was significantly ($p < .05$) less than in the proximal halves of these two arteries.

Publications: Roberts, W.C., and Virmani, R.: The amount of coronary narrowing in the 4 major epicardial coronary arteries in unstable angina pectoris: The quantitative approach. Am J Medicine (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03142-01 PA

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Quantitation of Coronary Arterial Narrowing at Necropsy in Acute Transmural Myocardial Infarction; An Analysis of 27 Patients and Comparison of Findings to Those in 22 Control Subjects

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

PI: William C. Roberts, Chief, Pathology Branch, NHLBI
Other: Ancil A. Jones, Clinical Associate, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A quantitative analysis of the degree and extent of coronary arterial narrowing by atherosclerotic plaques in the entire lengths of each of the 4 major coronary arteries in 27 necropsy patients with acute transmural myocardial infarction is described at necropsy for the first time. Of the 1403 five-mm segments examined in the 27 study patients, 484 (34%) were 76-100% narrowed in cross-sectional area by atherosclerotic plaques (controls = 3%); 528 (38%) were 51-75% narrowed (controls = 25%); 319 (23%) were 26-50% (controls = 44%) and only 72 segments (5%) were ≥ 25% narrowed (controls = 28%).

1015

Project Description: A quantitative analysis of the degree and extent of coronary arterial narrowing by atherosclerotic plaques in the entire lengths of each of the 4 major coronary arteries in 27 necropsy patients with acute transmural myocardial infarction is described at necropsy for the first time. A total of 1403 five-mm segments of the left main, left anterior descending, left circumflex and right coronary arteries were examined in 27 patients with acute transmural myocardial infarction and the observations in them were compared to those made from examination of 1002 five-mm segments of major epicardial coronary artery in 22 control subjects. An average of 26 cm (52 five-mm segments) of coronary artery were examined from each patient and an average of 22.5 cm (45 five-mm segments) from each control subject. Of the 1403 five-mm segments examined in the 27 study patients, 484 (34%) were 76-100% narrowed in cross-sectional area by atherosclerotic plaques (controls = 3%); 528 (38%) were 51-75% narrowed (controls = 25%); 319 (23%) were 26-50% (controls = 44%) and only 72 segments (5%) were $\geq$ 25% narrowed (controls = 28%). The amount of severe (<75%) narrrowing of the right, left anterior descending and left circumflex coronary arteries by atherosclerotic plaques was similar. Additionally, the amount of severe (> 75%) narrowing in the distal one-half of the right, left anterior descending and left circumflex coronary arteries was similar to that in the proximal halves of these 3 arteries. The number of 5-mm coronary segments narrowed 76 to 100% in cross-sectional area in the study patients was not affected by the patient's age at death, by the presence or absence of a history of previous angina pectoris or healed myocardial infarction, or by the weight of the heart. The men, however, had significantly ($p < .05$) more 5-mm segments of coronary artery > 75% narrowed in cross-sectional area than did the women and the patients with associated transmural left ventricular scars had significantly more coronary segments >75% narrowed than did the patients without transmural scars.

Publications: Roberts, W.C., and Jones, A.A.: Quantitation of coronary arterial narrowing at necropsy in acute transmural myocardial infarction: An analysis of 27 patients and comparison of findings to those in 22 control subjects. Circulation (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03143-01 PA

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Coronary Dilated Cardiomyopathy: Qualitation and Quantitation of Coronary Arterial Narrowing and of Left Ventricular Myocardial Scarring in 18 Necropsy Patients

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

PI: Renu Virmani, Staff Fellow, Pathology Branch, NHLBI
Other: William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A qualitative and quantitative analysis is described of the amount of ventricular wall myocardial scarring and the degree and extent of coronary arterial narrowing by atherosclerotic plaques in the entire lengths of each of the 4 major epicardial coronary arteries in 18 necropsy patients with healed transmural myocardial infarcts, chronic congestive heart failure and cardiomegaly. Of 1012 five-mm segments of the 4 major epicardial coronary arteries examined in the 18 study patients (average 54 segments per patient), 298 segments (29%) were 76 to 100% narrowed in cross-sectional area by atherosclerotic plaques (in 16 control subjects = 6%), 370 (37%) were 51 to 75% narrowed (controls = 35%), 227 (23%) were 26 to 50% narrowed (controls = 43%), and 117 (11%) were 0 to 25% narrowed (controls = 16%).

1017

Project Description: A qualitative and quantitative analysis is described of the amount of ventricular wall myocardial scarring and the degree and extent of coronary arterial narrowing by atherosclerotic plaques in the entire lengths of each of the 4 major epicardial coronary arteries in 18 necropsy patients with healed transmural myocardial infarcts, chronic congestive heart faliure and cardiomegaly. In all 18 patients, the healed infarcts involved greater than 40% of the left ventricular wall, all had very dilated right and left ventricular cavities, all had hearts weighing more than 450 gms (avg = 587), all had intractable congestive heart failure for longer than 3 months (avg = 2.3 years), and half had intraventricular mural thrombi. Of 1012 five-mm segments of the 4 major epicardial coronary arteries examined in the 18 study patients (average 54 segments per patient), 298 segments (29%) were 76 to 100% narrowed in cross-sectional area by atherosclerotic plaques (in 16 control subjects = 6%), 370 (37%) were 51 to 75% narrowed (controls = 35%), 227 (23%) were 26 to 50% narrowed (controls = 43%), and 117 (11%) were 0 to 25% narrowed (controls = 16%). The amount of severe (greater than 75% narrowing of the right, left anterior descending and left circumflex coronary arteries was similar in the 18 study patients. The left main coronary artery was not severely narrowed in any patient. The amount of severe (greater than 75%) narrowing in the distal one-half of the right, left anterior descending and left circumflex coronary arteries was similar to that in the proximal halves of these 3 arteries. The percent of 5-mm segments of coronary artery narrowed 76 to 100% in cross-sectional area in the 18 patients was similar in the patients 45 years of age and under and in those over 45 years of age, in those with compared to those without left ventricular aneurysm and in those with one compared to those with more than one acute myocardial infarct which healed. The patients with systemic hypertension, however, had more severe narrowing of the coronary arteries than did those with normal systemic arterial pressures.

Publications: Virmani, R., and Roberts, W.C. Coronary dilated cardiomyopathy: Qualitation and quantitation of coronary arterial narrowing and of left ventricular myocardial scarring in 18 necropsy patients. Submitted to Amer J Medicine

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03144-01 PA

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Non-Fatal Healed Transmural Myocardial Infarction in Fatal Non-Cardiac Disease: Qualification and Quantification of Coronary Arterial Narrowing and of Left Ventricular Scarring in 18 Necropsy Patients

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

PI: Renu Virmani, Staff Fellow, Pathology Branch, NHLBI
Other: William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Pathology Branch

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A qualitative and quantitative analysis of the amount of myocardial scarring and the degree and extent of coronary arterial narrowing by atherosclerotic plaque in the entire lengths of each of the 4 major epicardial coronary arteries is described in 18 necropsy patients with healed transmural myocardial infarcts and death from a non-cardiac condition. Of 924 five-mm segments examined in the 18 study patients, 292 (31%) were 76-100% narrowed in cross-sectional area by atherosclerotic plaques (controls = 5%); 319 (35%) were 51 to 75% narrowed (controls = 34%); 211 (23%) were 26 to 50% narrowed (controls = 44%), and 102 (11%) were 0 to 25% narrowed (controls = 17%).

1019

Project Description: A qualitative and quantitative analysis of the amount of myocardial scarring and the degree and extent of coronary arterial narrowing by atherosclerotic plaque in the entire lengths of each of the 4 major epicardial coronary arteries is described in 18 necropsy patients with healed transmural myocardial infarcts and death from a non-cardiac condition. An average of 30% of the basal half and 38% of the apical half of the left ventricular wall was scarred. The 9 patients without histories of acute myocardial infarcts (average 10 years before death) had left ventricular scars similar in size to the 9 patients with clinically silent myocardial infarcts. An average of 26 cm (51 five-mm segments) of coronary artery were examined from each patient and 25 cm (49 five-mm segments) from each of 19 control subjects. Of 924 five-mm segments examined in the 18 study patients, 292 (31%) were 76-100% narrowed in cross-sectional area by atherosclerotic plaques (controls = 5%); 319 (35%) were 51 to 75% narrowed (controls = 34%); 211 (23%) were 26 to 50% narrowed (controls = 44%), and 102 (11%) were 0 to 25% narrowed (controls = 17%). The amount of severe (> 75% narrowing was similar (25%) in the left anterior descending and left circumflex coronary arteries; the right was the most severely narrowed; the left main was not severely narrowed in any patient. The amount of severe (> 75%) narrowing in the proximal and distal halves of each of the right, left anterior descending and left circumflex coronary arteries was similar. The amount of severe (> 75%) coronary narrowing was not affected by patients' age at death or heart weight, but the amount of severe (> 75%) narrowing was greater in patients with compared to those without systemic hypertension and in patients with compared to those without a history of acute myocardial infarction.

Publications: Virmani, R., and Roberts, W.C.: Non-fatal healed transmural myocardial infarction in fatal non-cardiac disease: Qualification and quantification of coronary arterial narrowing and of left ventricular scarring in 18 necropsy patients. Submitted to Brit Heart J

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03145-01 PA

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Comparison of Coronary Narrowing in Coronary Heart Disease by Cineangiography to That Observed at Necropsy

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

PI: Ernest J. Arnett, Staff Associate, Pathology Branch, NHLBI

Other: Jeffrey M. Isner, Staff Associate, Pathology Branch, NHLBI
David Redwood, Senior Investigator, Cardiology Branch, NHLBI
Kenneth M. Kent, Senior Investigator, Cardiology Branch, NHLBI
William Baker, Chief, Cardiovascular Division, National Naval Medical Center, Bethesda, Maryland
Harold Ackerstein, Senior Staff, Radiology Department, Clinical Center, National Institutes of Health
William C. Roberts, Chief, Pathology Branch, NHLBI, NIH

COOPERATING UNITS (if any)
Cardiology Branch, NHLBI, Cardiovascular Division, National Naval Medical Center, and Radiology Department, Clinical Center

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Of 10 patients with fatal coronary heart disease undergoing coronary angiography 0-69 days (average 21) before necropsy, the amount of narrowing in 61 coronary arteries observed angiographically (diameter reduction) during life by three angiographers was compared to that observed histologically (cross-sectional area) at necropsy. Of 11 coronary arteries or their subdivisions narrowed 0-50% in cross-sectional area histologically, none were underestimated angiographically; of eight narrowed 51-75% histologically, seven had been underestimated, and of 42 narrowed 76-100% histologically, 17 (40%) were underestimated angiographically.

1021

PHS-6040
(Rev. 10-76)

Project Description: Of 10 patients with fatal coronary heart disease undergoing coronary angiography 0-69 days (average 21) before necropsy, the amount of narrowing in 61 coronary arteries observed angiographically (diameter reduction) during life by three angiographers was compared to that observed histologically (cross-sectional area) at necropsy. No overestimations of the degree of narrowing were made angiographically. Of 11 coronary arteries or their subdivisions narrowed 0-50% in cross-sectional area histologically, none were underestimated angiographically; of eight narrowed 51-75% histologically, seven had been underestimated, and of 42 narrowed 76-100% histologically, 17 (40%) were underestimated angiographically. The coronary atherosclerotic plaquing was diffuse (> 25% cross-sectional area narrowing) in 90% of 467 five-mm long segments of coronary artery examined (24 cm per patient) and this diffuseness of the atherosclerotic process appears to be the major reason for angiographic underestimation of coronary narrowings.

Publications: Arnett, E.N., Isner, J.M., Redwood, D.R., Kent, K.M., Baker, W.P., Ackerstein, H., and Roberts, W.C.: Comparison of coronary heart disease by cineangiography to that observed at necropsy. Annals of Intern Med. (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03146-01 PA

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

The Heart in Diabetes Mellitus as Viewed from a Morphologic Perspective

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

PI: Bruce F. Waller, Clinical Associate, Pathology Branch, NHLBI
Other: Pasquale J. Palumbo,
J. T. Lie,
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Pathology Branch

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Of 229 necropsy patients with diabetes mellitus (DM) with onset after age 30 years, 164 had clinical evidence of coronary heart disease (CHD) and 65 did not. The observations in the 229 diabetic patients were compared to those in 183 age-sex-matched non-diabetic patients with CHD. The average number of 3 major (right, left anterior descending, left circumflex) coronary arteries per patient narrowed > 75% in cross-sectional area by atherosclerotic plaque was similar in the DM patients with or without clinical CHD and both groups were similar to the non-DM patients with CHD: (DM - CHD vs. DM + CHD vs. CHD - DM: 2.4/3.0: 2.6/3.0: 2.5/3.0).

1023

PHS-6040
(Rev. 10-76)

Project Description: Of 229 necropsy patients with diabetes mellitus (DM) with onset after age 30 years, 164 had clinical evidence of coronary heart disease (CHD) and 65 did not. The observations in the 229 diabetic patients were compared to those in 183 age-sex-matched non-diabetic patients with CHD. The average number of 3 major (right, left anterior descending, left circumflex) coronary arteries per patient narrowed > 75% in cross-sectional area by atherosclerotic plaque was similar in the DM patients with or without clinical CHD and both groups were similar to the non-DM patients with CHD: (DM - CHD vs. DM + CHD vs. CHD - DM: 2.4/3.0: 2.6/3.0: 2.5/3.0). The percent of each of the 3 major coronary arteries (right, left anterior descending, left circumflex) narrowed > 75% in cross-sectional area by atherosclerotic plaque was similar in the 2 DM study groups and in the non-DM patients. Furthermore, the percent of proximal and distal halves of these arteries narrowed to this degree was similar. The amount of severe (> 75%) narrowing of the left main (LM) coronary artery was significantly greater ($p < .01$) in the DM patients with or without CHD than in non-DM patients with CHD (13% vs 6%). The DM + CHD patients also had significantly ($p < .05$) more severe left main narrowing than did the DM - CHD patients (14% vs. 11%). Significant differences in frequency of 7 clinical or morphologic parameters were noted when comparing the DM patients with CHD to the non-DM patients with CHD: the DM patients had a higher frequency of obesity (3.6x), healed myocardial infarction (1.9x), acute myocardial infarction (1.2x), left main coronary narrowing > 75% in cross-sectional area, and a lower frequency of cigarette smoking (31% less), mean total serum cholesterol (11% less) and sudden coronary death (46% less). The duration of, the type of treatment for, and the level of fasting blood glucose in the DM patients with onset of DM after age 30 years appeared to have no significant effect on the amount of severe (> 75%) coronary narrowing observed.

Publications: Waller, B.F., Palumbo, P.J., Lie, J.T., and Roberts, W.C.: The Heart in Diabetes Mellitus as Viewed from a Morphologic Perspective. In Diabetes and the Heart, Futura Publishing Co., Mt. Kisco, N.Y., 1979 (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03147-01 PA

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Bilateral Oophorectomy in Menstruating Women and Accelerated Coronary Atherosclerosis: An Unproved Connection

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

PI: William C. Roberts, Chief, Pathology Branch, NHLBI
Other: Alvaro A. Giraldo, Guest Worker, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, 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)

Although experimental studies in rabbits and in chickens suggest that total oophorectomy accelerates atherosclerosis in them and that estrogen has some protective effect (13), in humans premenopausal castration has not been conclusively demonstrated to accelerate coronary arterial atherosclerosis. This report analyzes previous reports of total oophorectomy in humans to show why accelerated atherosclerosis in them has not been proven.

1025

PHS-6040
(Rev. 10-76)

Project No. Z01 HL 03147-01 PA

Project Description: Although experimental studies in rabbits and in chickens suggest that total oophorectomy accelerates atherosclerosis in them and that estrogen has some protective effect (13), in humans premenopausal castration has not been conclusively demonstrated to accelerate coronary arterial atherosclerosis. This paper analyzes previous reports of total oophorectomy in humans to show why accelerated atherosclerosis in them has not been proven.

Publications: Roberts, W.C., and Giraldo, A.A.: Bilateral Oophorectomy in Menstruating Women and Accelerated Coronary Atherosclerosis: An Unproved Connection. Am J Med (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03148-01 PA

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Thrombocytosis, Coronary Thrombosis and Acute Myocardial Infarction

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

PI: Renu Virmani, Staff Fellow, Pathology Branch, NHLBI
Other: Mark A. Popovsky, Resident in Pathology, Laboratory of Pathology, NCI
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, 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 findings are described in a 22-year-old man with prolonged thrombocytosis and coronary and splenic arterial thrombi causing myocardial and splenic infarcts. The absence of preexistent extensive coronary atherosclerosis, the presence of thrombus in more than one epicardial artery and in multiple intramural coronary arteries, the presence of arterial thrombosis in a non-coronary artery (splenic), and the absence of another apparent cause of the arterial thromboses are evidences that the intraarterial clotting in our patient was related to the severe thrombocytosis.

1027

Project No. Z01 HL 03148-01 PA

Project Description: Clinical and morphologic findings are described in a 22-year-old man with prolonged thrombocytosis and coronary and splenic arterial thrombi causing myocardial and splenic infarcts. The absence of preexistent extensive coronary atherosclerosis, the presence of thrombus in more than one epicardial artery and in multiple intramural coronary arteries, the presence of arterial thrombosis in a non-coronary artery (splenic), and the absence of another apparent cause of the arterial thromboses are evidences that the intraarterial clotting in our patient was related to the severe thrombocytosis. Review of reported patients with vascular occlusion associated with thrombocytosis indicates that thrombi have infrequently been confirmed as the mechanism of the vascular occlusion. Although the frequency of vascular thrombi in patients with thrombocytosis has not been established, it is clear the vascular thrombosis can be a consequence of thrombocytosis and, as demonstrated by the present patient, that the coronary artery may be site of the vascular occlusion, a heretofore unconfirmed event.

Publications: Virmani, R., Popovsky, M.A., and Roberts, W.C.: Thrombocytosis and acute myocardial infarction. Am J Med (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03149-01 PA

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Frequency and Direction of Interatrial Shunting in Valvular Pulmonic Stenosis with Intact Ventricular Septum and without Left Ventricular Inflow or Outflow Obstruction: An Analysis of 127 Patients Treated by Valvulotomy

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

PI: William C. Roberts, Chief, Pathology Branch, NHLBI

Other: Richard J. Shemin, Surgical Associate, Surgery Branch, NHLBI

Kenneth M. Kent, Chief, Diagnostic Section, Cardiology Branch, NHLBI

COOPERATING UNITS (if any)

Surgery Branch, NHLBI and Cardiology Department, NHLBI

LAB/BRANCH

Pathology Branch

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

This report summarizes observations in 127 patients who underwent pulmonic valvulotomy for valvular pulmonic stenosis with intact ventricular septum and without obstruction to left ventricular inflow or outflow. Of the 127 patients, 30 (24%) preoperatively by dye-dilution curves had shunting at the atrial level: in 19 (63%), the shunt was right to left and in the other 11 (27%), entirely left to right. The patients with right-to-left interatrial shunts had severe pulmonic valve stenosis (average peak systolic pressure gradient + 120 + 11 mm Hg) and small (average diameter 1.1 + 0.1 cm) sized defects in the atrial septum (patent foramen ovale). In contrast, the patients with left-to-right shunts had mild to moderate pulmonic valve stenosis (average peak systolic pressure gradient = 60 + 5 mm Hg) and relatively large (average diameter = 2.8 + 0.1 cm) defects in the atrial septum (true atrial septal defect).

1029

PHS-6040
(Rev. 10-76)

Project Description: This report summarizes observations in 127 patients who underwent pulmonic valvulotomy for valvular pulmonic stenosis with intact ventricular septum and without obstruction to left ventricular inflow or outflow. Of the 127 patients, 30 (24%) preoperatively by dye-dilution curves had shunting at the atrial level: in 19 (63%), the shunt was right to left and in the other 11 (27%), entirely left to right. The patients with right-to-left interatrial shunts had severe pulmonic valve stenosis (average peak systolic pressure gradient = 120 ± 11 mm Hg) and small (average diameter 1.1 ± 0.1 cm) sized defects in the atrial septum (patent foramen ovale). In contrast, the patients with left-to-right shunts had mild to moderate pulmonic valve stenosis (average peak systolic pressure gradient = 60 ± 5 mm Hg) and relatively large (average diameter = 2.8 ± 0.1 cm) defects in the atrial septum (true atrial septal defect). The patients with right-to-left interatrial shunts had no significant differences in right versus left atrial pressures. The patients with left-to-right interatrial shunts, however, had left atrial pressures significantly greater than right atrial pressures (7 ± 0.5-vs-5 ± 0.5, $p < .05$). No significant differences were found in ventricular end-diastolic pressures.

Publications: Roberts, W.C., Shemin, R.J., and Kent, K.M.: Frequency and direction of interatrial shunting in valvular pulmonic stenosis with intact ventricular septum and without left ventricular inflow or outflow obstruction: An analysis of 127 patients treated by valvulotomy. Submitted to Amer J Cardiol

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03150-01 PA

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Left and Right Ventricular Myocardial Infarction in Idiopathic Dilated Cardiomyopathy

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

PI: Jeffrey M. Isner, Staff Associate, Pathology Branch, NHLBI

Other: Renu Virmani, Staff Fellow, Pathology Branch, NHLBI
Samuel B. Itscoitz, Staff Cardiologist, Georgetown University School of Medicine
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

Georgetown University School of Medicine, Washington, D.C.

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, 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 findings are described in a 62-year-old woman who had idiopathic dilated cardiomyopathy and at necropsy was found to have unsuspected scarring of the right and left ventricles. Although extensive scarring of the left ventricle has been occasionally described in such patients, it is uncommon. Right ventricular scarring, however, has never before been described. Shortly prior to death this patient manifested signs of pericardial heart disease, yet at necropsy the pericardium was normal. This report points out the manner in which right ventricular scarring in patients with idiopathic dilated cardiomyopathy may mimic pericardial heart disease.

1031

PHS-6040
(Rev. 10-76)

Project Description: Clinical and morphologic findings are described in a 62-year-old woman who had idiopathic dilated cardiomyopathy and at necropsy was found to have unsuspected scarring of the right and left ventricles. Although extensive scarring of the left ventricle has been occasionally described in such patients, it is uncommon. Right ventricular scarring, however, has never before been described. Shortly prior to death this patient manifested signs of pericardial heart disease, yet at necropsy the pericardium was normal. This report points out the manner in which right ventricular scarring in patients with idiopathic dilated cardiomyopathy may mimic pericardial heart disease.

Publications: Isner, J.M., Virmani, R., Itscoitz, S.B., and Roberts, W.C.: Left and right ventricular myocardial infarction in idiopathic dilated cardiomyopathy. Amer Heart J (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03151-01 PA

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Sudden Unexpected Death in Avid Dieters Using the Liquid-Protein-Modified-Fast Diet: Observations in 17 Patients and the Role of the Prolonged Q-T Interval

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

PI: Jeffrey M. Isner, Staff Associate, Pathology Branch, NHLBI

Other: Harold E. Sours, Medical Officer, Center for Disease Control, Atlanta, Georgia
Allen L. Paris, Medical Officer, Center for Disease Control, Atlanta, Georgia
Victor J. Ferrans, Medical Officer, Pathology Branch, NHLBI
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)
Center for Disease Control, Atlanta, Georgia

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, 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 findings are described in 17 patients who died suddenly and unexpectedly during or shortly following use of the liquid-protein-modified-fast diet. Of the 17 patients, 16 were women, most were young (avg age = 37 years), and most lost a massive amount of weight (avg = 41 kg or 35% of their pre-diet weight) over a short period of time (avg = 5 months). Eight had one or more episodes of syncope. Multiple-lead electrocardiograms were recorded in 10 patients; all had normal sinus rhythm; all had episodes of ventricular tachycardia; nine and possibly 10 patients had prolongation of the Q-T interval unassociated with the recognized causes of Q-T interval prolongation in at least seven of the nine patients; and nine had diminished amplitude of the QRS complexes ("low voltage").

1033

Project Description: Clinical and morphologic findings are described in 17 patients who died suddenly and unexpectedly during or shortly following use of the liquid-protein-modified-fast diet. Of the 17 patients, 16 were women, most were young (avg age = 37 years), and most lost a massive amount of weight (avg = 41 kg or 35% of their pre-diet weight) over a short period of time (avg = 5 months). Eight had one or more episodes of syncope. Multiple-lead electrocardiograms were recorded in 10 patients; all had normal sinus rhythm; all had episodes of ventricular tachycardia; nine and possibly 10 patients had prolongation of the Q-T interval unassociated with the recognized causes of Q-T interval prolongation in at least seven of the nine patients; and nine had diminished amplitude of the QRS complexes ("low voltage"). Histologic study of left ventricular myocardium in 14 patients disclosed attenuated myocardial fibers in 12, increased lipofuscin pigment in 11, and mononuclear-cell myocarditis in one. Similar histologic findings, however, also were found in 16 cachetic control subjects studied in similar fashion, but electrocardiograms in them showed no prolongation of Q-T intervals or episodes of ventricular tachycardia. Thus, semi-starvation, particularly in the face of antecedent obesity, is a cause of acquired Q-T interval prolongation, and repeated electrocardiograms are recommended in patients on semi-starvation diets for treatment of obesity.

Publications: Isner, J.M., Sours, H.E., Paris, A.L., Ferrans, V.J., and Roberts, W.C.: Sudden unexpected death in avid dieters using the liquid-protein-modified-fast diet: Observations in 17 patients and the role of the prolonged Q-T interval. Circulation (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03152-01 PA

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Complications of the Intra-Aortic Balloon Counterpulsation Device: Clinical and Morphologic Observations in 45 Necropsy Patients

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

PI: Jeffrey M. Isner, Staff Associate, Pathology Branch, NHLBI

Other: Steven R. Cohen, Medical Student, Pathology Branch, NHLBI
Renu Virmani, Staff Fellow, Pathology Branch, NHLBI
Walter Lawrinson, Director of Clinical Pathology, Washington Hospital Center, D.C.
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

Clinical Pathology, Washington Hospital Center, D.C.

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Of 45 patients who died after insertion of an intra-aortic balloon assist device (IABAD) and who were studied at necropsy, 16 (36%) were found to have one or more complications (total 20) related to use of the device. The 20 complications consisted of dissection of the aorta and/or its distal branches (9), arterial perforation (3), arterial thrombi (3), arterial emboli (3) extremity ischemia (1), and local wound infection (1).

1035

PHS-6040
(Rev. 10-76)

Project Description: Of 45 patients who died after insertion of an intra-aortic balloon assist device (IABAD) and who were studied at necropsy, 16 (36%) were found to have one or more complications (total 20) related to use of the device. The 20 complications consisted of dissection of the aorta and/or its distal branches (9), arterial perforation (3), arterial thrombi (3), arterial emboli (3), extremely ischemia (1), and local wound infection (1). Of the 9 patients with arterial dissection, none were diagnosed or suspected before necropsy. Of the total 20 complications, only 4 (20%) were suspected before death. Although the operating team frequently encountered no difficulty at the time of insertion of the device, 12 of the 20 complications were a direct result of insertion of the IABAD. In 2 patients in whom insertion of the IABAD caused dissection of the aorta, hemodynamic improvement occurred for 2 and 3 days respectively despite the fact that the "intra-aortic" balloon (as well as the catheter) was not located in the true lumen of the aorta. Thus, clinical evaluation of complications related to use of the IABAD underestimates their frequency. Most complications are a consequence of insertion of the device, not consequences of its being in place.

Publications: Isner, J.M., Cohen, S.R., Virmani, R., Lawrinson, W., and Roberts, W.C.: Complications of the intra-aortic balloon counterpulsation device: Clinical and morphologic observations in 45 necropsy patients. Am J Cardiology (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03153-01 PA

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
The Sucking Action of the Left Ventricle: Demonstration of a Physiologic Principle by a Gun-shot Wound Penetrating Only the Right Side of the Heart

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

PI: William C. Roberts, Chief, Pathology Branch, NHLBI
Other: William J. Brownlee, Pathologist, DC Medical Examiners Office, NHLBI
Ancil A. Jones, Clinical Associate, Pathology Branch, NHLBI
James L. Luke, Chief, Medical Examiner, District of Columbia Medical Examiners Office, NHLBI

COOPERATING UNITS (if any)
DC Medical Examiners Office, NHLBI

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

This report describes a man who died following a gun-shot wound which entered the right atrium and exited the right ventricle without entering the cardiac septa or the left side of the heart. At necropsy, the left atrial appendage was found to be inverted and invaginated into the mitral orifice. The invagination of the left atrial appendage is viewed as anatomic evidence that a negative left ventricular pressure was created as the left ventricular volume rapidly fell from right-sided cardiac exsanguination.

1037

PHS-6040
(Rev. 10-76)

Project Description: This report describes a man who died following a gun-shot wound which entered the right atrium and exited the right ventricle without entering the cardiac septa or the left side of the heart. At necropsy, the left atrial appendage was found to be inverted and invaginated into the mitral orifice. The invagination of the left atrial appendage is viewed as anatomic evidence that a negative left ventricular pressure was created as the left ventricular volume rapidly fell from right-sided cardiac exsanguination. Previously reported experiments in non-human animals demonstrating the sucking (negative pressure) action of the left ventricle during ventricular diastole are summarized. The prerequisite for creation of a negative pressure in the ventricles during diastole is an extreme diminution in left ventricular volume, in our patient by right-sided cardiac bleeding. Explanations other than a vaccum effect of the left ventricle during diastole cannot explain the inversion and invagination of the left atrial appendage in our patient.

Publications: Roberts, W.C., Brownlee, W.J., Goldman, M.H., and Luke, J.L.: The sucking action of the left ventricle: Demonstration of a physiologic principle by a gun-shot wound penetrating only the right side of the heart. Am J Cardiology: 1234-1237, 1979

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03154-01 PA

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Description of a Dry-Ice Container Mounted on a Microtome for Continuous Cooling of Tissue-Containing Paraffin Blocks During Sectioning

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

PI: Filippina Giacometti, Histopathology Technician, Pathology Branch, NHLBI
Other: William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Because of certain deficiencies of presently available methods of cooling tissue-containing paraffin blocks during or immediately before their being cut, a new method of cooling the blocks was developed. It consists of "bathing" the paraffin block during the process of cutting in cooled air evaporated from dry ice contained in a container which is mounted on a microtome and which overhangs the mounted paraffin block. Perforations in the floor of the overhanging portion of the container and closure of the top of the container allows evaporation of the dry ice over the mounted paraffin block with uniform cooling of the block. The quality of histologic sections has been improved by this new method of cooling of paraffin blocks.

1039

Project Description: Because of certain deficiencies of presently available methods of cooling tissue-containing paraffin blocks during or immediately before their being cut, a new method of cooling the blocks was developed. It consists of "bathing" the paraffin block during the process of cutting in cooled air evaporated from dry ice contained in a container which is mounted on a microtome and which overhangs the mounted paraffin block. Perforations in the floor of the overhanging portion of the container and closure of the top of the container allows evaporation of the dry ice over the mounted paraffin block with uniform cooling of the block. The quality of histologic sections has been improved by this new method of cooling of paraffin blocks.

Publications: Giacometti, F., and Roberts, W.C.: Description of a dry-ice container mounted on a microtome for continuous cooling of tissue-containing paraffin blocks during sectioning. Stain Technology (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03155-01 PA

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Cardiac Lesions Induced by Minoxidil

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	Food and Drug Administration	
	Tibor Balazs	Food and Drug Administration	
	Robert Young	Food and Drug Administration	
	Francis L. Earl	Food and Drug Administration	
	Stephen Krop	Food and Drug Administration	

COOPERATING UNITS (if any)
Food and Drug Administration, Washington, D. C.

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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)

Lesions produced in beagle dogs by the oral administration of 0.5 - 3.0 mg/kg of minoxidil, a vasodilating antihypertensive agent, consist of: 1) focal, superficial hemorrhages in epicardium and endocardium; 2) focal arteritis in small coronary vessels, and 3) left ventricular papillary muscle necrosis.

1041

PHS-6040
(Rev. 10-76)

Project No. Z01 HL 03155-01 PA

Project Description: Minoxidil, a vasodilating antihypertensive drug, was given orally in doses of 0.5, 1.0, or 3.0 mg/kg to adult Beagle dogs on 2 consecutive days. Within 2 hr. of administration, each of these doses produced hypotension and a marked tachycardia which persisted for as long as 24 hr. after the second dose of minoxidil. Necropsies performed 24 hr. after the second dose revealed focal, superficial areas of epicardial or endocardial hemorrhage in dogs given each of the three dose levels. Hemorrhage was associated with a mild inflammatory reaction and was not limited to the right atrium. Focal arteritis, characterized by extravasation of blood and by focal accumulation of erythrocytes and fibrin-like material in the walls of small coronary arteries, occurred in three hearts from the 3-mg/kg dose group. Myocardial necrosis was noted in one of eight dogs given the minimal pharmacologic dose of 0.5 mg/kg, and in five of eight dogs in each of the groups given 1 or 3 mg/kg. Necrosis was most frequent in the left ventricular papillary muscles, particularly the posterior one. An ischemic origin of the necrosis is suggested by the localization of the lesions and by the pharmacologic effects of minoxidil.

Publications: Herman, E. H., Balazs, T., Young, R., Earl, F. J., Krop, S., and Ferrans, V. J.: Acute cardiomyopathy induced by the vasodilating antihypertensive agent minoxidil. Toxicol. Appl. Pharmacol. 47: 493-503, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03156-01 PA

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Tubuloreticular Structures in Myocardium

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

PI:	Victor J. Ferrans	Chief, Ultrastructure Section	PA	NHLBI
Others:	Paul J. Boor	Guest Worker, Ultrastructure Section	PA	NHLBI
	Michael Jones	Senior Surgeon, Clinic of Surgery		NHLBI
	Oichi Kawanami	Visiting Expert, Ultrastructure Section		NHLBI
	K.-U. Thiedemann	Guest Worker, Ultrastructure Section	PA	NHLBI
	E. H. Herman	Div. of Drug Biology, Food and Drug Administration		
	W. C. Roberts	Chief, Pathology Branch	PA	NHLBI

COOPERATING UNITS (if any)

Clinic of Surgery, National Heart, Lung, and Blood Institute
Division of Drug Biology, Food and Drug Administration, Washington, D. C.

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

This report describes the occurrence of tubuloreticular structures in cardiac tissue in 5 of 169 patients with diverse types of heart disease. Tubuloreticular structures were found in endothelial cells and fibroblasts, but not in cardiac muscle cells. They are derived from cisterns of endoplasmic reticulum, and they do not have specific diagnostic significance.

1043

PHS-6040
(Rev. 10-76)

Project No. Z01 HL 03156-01 PA

Project Description: Tubuloreticular structures were observed in cardiac tissue obtained either by biopsy or by operative resection in five (3%) of 169 patients with diverse conditions. Two of these five patients had congenital heart disease; one, aortic valvular disease, and two had cardiomyopathy, which was associated with thyrotoxicosis in one patient and with polymyositis and chronic alcoholism in the other. Tubuloreticular structures were composed of curved or undulating tubules that measured from 200 to 300 Å in diameter, branched extensively, and formed net-like masses either within cisterns of endoplasmic reticulum or within the perinuclear cistern. They occurred in endothelial or fibroblast-like cells, but not in cardiac muscle cells. These structures are known to occur in a wide variety of normal and abnormal animal and plant cells. They are formed through a specialized type of deformation of the membranes of the endoplasmic reticulum. Their precise significance is unknown. Criteria are given for the distinction between tubuloreticular structures and other types of morphologically similar structures that have been described in muscle cells, including aggregates of tubules of sarcoplasmic reticulum, tubules derived from the inner nuclear membranes, annulate lamellae, and highly organized arrays of developing T tubules.

Publications: Boor, P. J., Ferrans, V. J., Jones, M., Kawanami, O., Thiedemann, K-U., Herman, E. H., and Roberts, W. C.: Tubuloreticular structures in myocardium. An ultrastructural study. J. Mol. Cell. Cardiol. (In press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03157-01 PA

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Nuclear Inclusions in Alveolar Epithelium in Fibrotic Lung Disorders

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

PI:	Oichi Kawanami	Visiting Expert	PA	NHLBI
Others:	Victor J. Ferrans	Chief, Ultrastructure Section	PA	NHLBI
	J. D. Fulmer	Staff Investigator, Pulmonary Branch		NHLBI
	R. G. Crystal	Chief, Pulmonary Branch		NHLBI

COOPERATING UNITS (if any)

Pulmonary Branch, NHLBI

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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)

Nuclear inclusions were found in a small percentage of cuboidal alveolar epithelial cells in 9 of 19 patients with fibrotic lung disorders. These inclusions consisted of masses of tubules and resembled nuclear tubules which occur in other cell types under conditions of rapid growth or specific hormonal stimulation.

1045

PHS-6040
(Rev. 10-76)

Project No. Z01 HL 03157-01 PA

Project Description: Ultrastructural study of pulmonary biopsies of patients with fibrotic lung disease disclosed the presence of nuclear inclusions in one per cent or less of cuboidal alveolar epithelial cells in 9 of 19 patients, including 6 of 12 patients with idiopathic pulmonary fibrosis, 2 of 3 patients with collagen-vascular diseases, and 1 of 3 patients with sarcoidosis. Nuclear inclusions were not observed by ultrastructural study in 5 control patients. The inclusions consisted of masses or aggregates of tubules which probably were derived from the inner nuclear membranes. These tubules were smooth-walled, showed branchings and bifurcations, were composed of single trilaminar membranes, usually had a clear content, and ranged from 500 to 1,000 Å in diameter. They resembled nuclear tubules which occur in other cell types under conditions of rapid growth or specific hormonal stimulation.

Statistically significant differences between the groups of patients with and without nuclear inclusions in cuboidal alveolar epithelial cells were not found with respect to: smoking history; degree of fibrosis in the lung biopsy specimen; or the degree of pulmonary physiologic impairment. However, the average age of the patients having nuclear inclusions was significantly greater than that of patients not having nuclear inclusions. In addition, the frequency of indentations in the nuclei of cuboidal alveolar epithelial cells was greater in patients with nuclear inclusions than in patients without nuclear inclusions. Highly significant correlations were observed between the presence of nuclear inclusions and the presence of: 1) anchoring fibrils and hemidesmosomes along the basal surfaces of alveolar epithelial cells, and 2) multilayering of the alveolar epithelium.

Publications: Kawanami, O., Ferrans, V. J., Fulmer, J. D. and Crystal, R. G.: Nuclear inclusions in alveolar epithelium of patients with fibrotic lung disorders. Am. J. Pathol. 94: 301-322, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03158-01 PA

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Ultrastructure of Pulmonary Mast 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	PA	NHLBI
	J. D. Fulmer	Staff Investigator, Pulmonary Branch		NHLBI
	R. G. Crystal	Chief, Pulmonary Branch		NHLBI

COOPERATING UNITS (if any)
Pulmonary Branch

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, 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)

Qualitative and quantitative electron microscopic studies disclosed extensive alterations in pulmonary mast cells in patients with fibrotic lung disorders. Mast cells were increased in number and showed evidence of degranulation and of migration through the alveolar epithelial cell layer into alveolar lumina. These phenomena may be of importance in the pathogenesis of the functional abnormalities and the continuing alveolar injury observed in fibrotic lung disorders.

1047

Project No. Z01 HL 03158-01 PA

Project Description: The topographic distribution, population density and ultrastructural features of metachromatic cells (mast cells and basophilic leukocytes) were studied in lung biopsies from 5 control patients and 17 patients with fibrotic lung disorders. The great majority of metachromatic cells were mast cells. The average number of metachromatic cells per mm^2 of tissue section was much larger in patients with fibrotic lung disorders (45.8 ± 6.5) than in control patients (2.6 ± 1.6). In control patients, mast cells were most frequently seen in subpleural and perivascular connective tissue. In contrast, the vast majority of mast cells in patients with fibrotic lung disorders was present in thickened, fibrous alveolar septa; mast cells also were found within the alveolar epithelial layer and alveolar lumina. The quantitative distribution of different types of mast cell granules differed in the two groups of patients: granules composed of scrolls were more frequent in control patients, and granules of the combined type (containing mixtures of different components within the same granule) were more frequent in patients with fibrotic lung disorders. Mast cells in the latter patients appeared to migrate through defects in the basement membrane into the epithelial layer and alveolar lumina; mast cells in these areas often showed reduced numbers of granules and disorganized granule content. These changes suggest that pulmonary parenchymal mast cells in fibrotic lung disorders undergo a chronic process of partial degranulation which differs from that found in anaphylaxis; this chronic release of mast cell products may contribute to the continuing alveolar injury and to the ventilation/perfusion inequalities observed in the fibrotic lung disorders.

Publications: Kawanami, O., Ferrans, V. J., Fulmer, J. D., and Crystal, R. G.: Ultrastructure of pulmonary mast cells in patients with fibrotic lung disorders. Lab. Invest. 40: 717-734, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03159-01 PA

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Anchoring Fibrils in Normal Canine Respiratory System

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

PI:	Oichi Kawanami	Visiting Expert	PA	NHLBI
Other:	Victor J. Ferrans	Chief, Ultrastructure Section	PA	NHLBI
	Ronald G. Crystal	Chief, Pulmonary Branch		NHLBI

COOPERATING UNITS (if any)

Pulmonary Branch, NHLBI

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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)

Anchoring fibrils, a distinct class of fibrils of extracellular connective tissue, were found in normal canine respiratory tree in association with: basal cells and special type cells of trachea and bronchi; ciliated cells and basal cells of bronchioles; and ductal cells, secretory cells and myoepithelial cells of tracheobronchial glands.

Anchoring fibrils reinforce the attachment of the epithelial basal lamina to the underlying connective tissues.

1049

Project No. Z01 HL 03159-01 PA

Project Description: Electron microscopic study of the normal canine respiratory tree disclosed the presence of anchoring fibrils, a distinct class of fibrils of extracellular connective tissue, in association with the following types of cells: basal cells and special type cells of trachea and bronchi; ciliated cells and basal cells of bronchioles; and ductal cells, secretory cells and myoepithelial cells of tracheobronchial glands. Anchoring fibrils in the normal respiratory system measured up to 6,000 Å in length and from 170 to 400 Å in thickness, and had a banding pattern that differed from that of collagen fibrils and connective tissue microfibrils. They formed arcs, the ends of which inserted into the basal lamina underlying the basal portions of the cells, often in the vicinity of hemidesmosomes. Anchoring fibrils decreased in number and size in the more distal portions of the respiratory tree, and were not found in alveolar septa. Anchoring fibrils in lung appeared similar to those described in other organs, but often were small and inconspicuous. The function of these structures is to reinforce the attachment of the epithelial basal lamina to the underlying connective tissues.

Publications: Kawanami, O., Ferrans, V. J., and Crystal, R. G.: Anchoring fibrils in normal canine respiratory system. Am. Rev. Resp. Dis. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03160-01 PA

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Ultrastructural Alterations in Porcine Valvular Heterografts

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	Biologist, Ultrastructure Section	PA	NHLBI
	M. E. Billingham	Department of Pathology, Stanford University School of Medicine		
	T. L. Spray	Staff Associate, Pathology Branch	PA	NHLBI
	W. C. Roberts	Chief, Pathology Branch	PA	NHLBI

COOPERATING UNITS (if any)
Stanford University School of Medicine, Stanford, California

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, 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)

Light and electron microscopic studies of structural alterations occurring in 30 porcine valvular heterografts after various periods of implantation in patients showed that alterations in valvular collagen are of crucial importance in determining the long-term durability of porcine valvular heterografts. The two most important complications in these heterografts are: 1) perforation of the leaflets, which is related to collagen breakdown and leads to valvular regurgitation, and 2) calcification of the leaflets, which involves the collagen and results in prosthetic valvular stenosis.

1051

Project No. Z01 HL 03160-01 PA

Project Description: Light and electron microscopic studies were made of structural alterations in 30 porcine valvular heterografts removed from 28 patients either at necropsy (12 patients) or at reoperation for replacement of a malfunctioning heterograft (16 patients). Early (< 2 months after implantation) morphological changes 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 (3 to 94 months after implantation) changes were characterized by progressively severe damage to the collagen, formation of aggregates of platelets on the valvular surfaces, lipid deposition, erosion of the surfaces, and development of calcific deposits.

Perforation of the leaflets, leading to prosthetic regurgitation, was a consequence of severe collagen breakdown. Conversely, 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 heterografts.

Publications: Ferrans, V. J., Boyce, S. W., Billingham, M. E., Spray, T. L., and Roberts, W. C.: Ultrastructural alterations in porcine valvular heterografts. Herz. (In press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03161-01 PA

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Infection of Porcine Valvular Heterografts

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:	Steven W. Boyce	Biologist, Ultrastructure Section	PA NHLBI
	M. E. Billingham	Department of Pathology, Stanford University School of Medicine	
	T. L. Spray	Staff Associate, Pathology Branch	PA NHLBI
	W. C. Roberts	Chief, Pathology Branch	PA NHLBI

COOPERATING UNITS (if any)

Stanford University School of Medicine, Stanford, California

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, 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)

Morphologic changes associated with bacterial infection are described in 4 porcine valvular heterografts. Observations made indicate that infection in these valves: 1) develops in the fibrin layer that covers the cusps; 2) can involve the collagen in the leaflets and, 3) is uncommonly associated with valve ring abcesses.

1053

PHS-6040
(Rev. 10-76)

Project No. Z01 HL 03161-01 PA

Project Description: Gross, histologic and ultrastructural changes associated with bacterial infection are described in 4 porcine valvular heterografts that had been in place in patients for periods of time ranging from 6 days to 28 months. In one patient, culture of the aortic tissue tag included in the heterograft container grew Mycobacterium chelonei; however, examination of the heterograft, recovered at necropsy 6 days after implantation, revealed small colonies of bacteria that differed morphologically from mycobacteria. A second heterograft was the site of staphylococcal infection associated with extensive destruction of collagen in the leaflets. Similar destruction was observed in a third heterograft, in which organisms were found on ultrastructural study even though bacterial cultures of the valve were negative. The fourth heterograft, from a patient who died of coronary embolism secondary to dislodgment of vegetative material, contained structures resembling lysed bacteria. Observations in our 4 patients and review of published reports of infection involving 43 other patients with porcine valvular heterografts indicates that infection in these valves: 1) develops in the fibrin layer that covers the cusps; 2) can involve the collagen in the leaflets, and 3) is uncommonly (3 patients) associated with valve ring abcesses.

Publications: Ferrans, V. J., Boyce, S. W., Billingham, M. E., Spray, T. L., and Roberts, W. C.: Infection of glutaraldehyde-preserved porcine valve heterografts. Am. J. Cardiol. 43: 1123-1136, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03162-01 PA

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Intramural Coronary Arteries in Dogs With Discrete Subaortic Stenosis

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

PI:	W. F. T. Muna	Visiting Fellow	PA	NHLBI
Others:	Victor J. Ferrans,	Chief, Ultrasturcture Section	PA	NHLBI
	J. E. Pierce	Chief, Section on Laboratory Animal Medicine and Surgery		
	W. C. Roberts	Chief, Pathology Branch	PA	NHLBI

COOPERATING UNITS (if any)

Section on Laboratory Animal Medicine and Surgery, NHLBI

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, 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)

Small intramural coronary arteries in dogs with discrete subaortic stenosis have a high incidence (> 50%) of narrowing by proliferation of intimal smooth muscle cells and by fibrosis involving all layers of the wall.

1055

PHS-6040
(Rev. 10-76)

Project No. Z01 HL 03162-01 PA

Project Description: Morphologic studies were made of small (100 μ to 1 mm diameter) intramural coronary arteries in 8 Newfoundland dogs with discrete fibrous ring type of subaortic stenosis. In each dog, histologic examination revealed a > 50% incidence of narrowing of these vessels by proliferation of smooth muscle cells in intima and by fibrosis involving intima, media and adventitia. These processes occurred to a varying extent and in different combinations along the length of a given vessel. Ultrastructural study showed that the basement membranes of endothelial cells were often reduplicated; those of smooth muscle cells were thickened. Other than this, smooth muscle cells appeared normal. The most striking abnormality involved intimal elastic tissue, which formed numerous small, scattered fibrils (< 1 μ in diameter) rather than discrete laminae separating layers of smooth muscle cells. Collagen fibers between smooth muscle cells were increased in number but were morphologically normal. The changes described resemble those in humans with hypertrophic cardiomyopathy and with discrete ring or tunnel types of subaortic stenosis. Thus, these changes are common to diseases in which subvalvular obstruction to left ventricular outflow results in ventricular hypertrophy.

Publications: Muna, W. F. T., Ferrans, V. J., Pierce, J. E. and Roberts, W. C.: Structure of intramural coronary arteries in dogs with discrete subaortic stenosis. Circulation 57 & 58 (Suppl. II): II-242, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03163-01 PA

PERIOD COVERED
October 1, 1978 to September 30, 1979

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
Other:	Michael Jones	Senior Surgeon, Clinic of Surgery		NHLBI
	W. C. Roberts	Chief, Pathology Branch	PA	NHLBI

COOPERATING UNITS (if any)
Clinic of Surgery

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A description of the pathologic changes that develop in aortocoronary bypass graft systems.

1057

PHS-6040
(Rev. 10-76)

Project No. Z01 HL 03163-01 PA

Project Description: A review is presented of the pathologic 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.

Problems related to operative judgement and technique include: 1) grafts that are either too short, producing excessive tension, or too long, predisposing to kinking; 2) distal anastomoses to coronary arteries that are too small or significantly narrowed by atherosclerotic plaques, and 3) proximal anastomoses that either do not have the proper angle between the vein and the aorta or that are made in areas of aortic atherosclerosis.

Early and late changes occur in vein grafts as responses of the venous tissue to the implantation procedure and to subsequent hemodynamic changes. Early changes consist of endothelial damage, non-occlusive platelet-fibrin aggregates, occlusive and non-occlusive thrombi and smooth muscle cell necrosis. Late changes include fibromuscular intimal proliferation and medial and adventitial fibrosis. In addition to these changes, which occur in a large majority of aortocoronary saphenous vein grafts, late alterations that develop in a small number of these implants include lipid deposition, aneurysmal dilatation and thrombotic occlusion with or without recanalization.

Pathologic changes that occur in the native coronary arteries after aortocoronary venous bypass procedures include: 1) progression of atherosclerosis in the regions of the coronary arteries proximal and distal to the graft site; 2) proximal thrombosis of the coronary artery (watershed effect); 3) extension of fibromuscular intimal thickening from the vein graft into the coronary artery either proximally or distally, and 4) dissection of the coronary artery.

Publications: Ferrans, V. J., Jones, Michael and Roberts, W. C.: The pathology of saphenous vein aortocoronary bypass grafts. Proceedings of the International Symposium on Selected Topics in Cardiac Surgery, Padova, Italy, 1979, P. G. Cevese, Ed. (In press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03164-01 PA

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Coronary Collateral Circulation

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

PI: Victor J. Ferrans Chief, Ultrastructure Section PA NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A review of the anatomy and physiology of the coronary collateral circulation.

1059

Project Description: Coronary collateral vessels are classified into three categories according to whether they provide anastomotic connections between a given coronary vessel and: 1) a cardiac chamber; 2) a systemic vessel other than a coronary artery, or 3) another coronary vessel. Two types of endomural vessels, arterioluminal vessels and Thebesian veins, provide direct communications between coronary vessels and the cardiac cavities. Three types of anastomoses can exist between coronary arteries and other systemic vessels: 1) small anastomoses between distal branches of the coronary arteries and systemic arteries, including: a) vasa vasorum of the great arteries and veins at the base of the heart, and b) vessels in the retrocardiac and retropericardial area; 2) anastomoses involving small vessels that develop in epicardial granulation tissue resulting either from pericardial disease or from surgical procedures designed to produce adhesions between the epicardium and pericardium or other tissues (omentum, lung, etc.), and 3) large anastomoses created surgically between the aorta or a systemic artery and a coronary artery. Anastomoses between two components of the coronary arterial tree are classified as intracoronary or intercoronary. Intracoronary anastomoses are those which connect two segments or branches of the same coronary artery. Intercoronary anastomoses are those which connect branches of two different coronary arteries. They constitute the most important alternate routes for delivery of blood to ischemic areas of myocardium.

Publications: Ferrans, V. J.: The significance of the coronary collateral circulation in myocardial oxygenation. Pract. Cardiol. 4: 49-62, 1978.

Project No. Z01 HL 03164-01 PA

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 03165-01 PA

PERIOD COVERED
October 1, 1978 to September 30, 1979

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:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A review summarizing our knowledge of cardiac ultrastructure in primary and secondary cardiomyopathies, with emphasis on problems of tissue diagnosis.

1061

PHS-6040
(Rev. 10-76)

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 Sekiguchi, M. and Olsen, E. J. G. (Eds.): Clinical and Experimental Aspects of Cardiomyopathy. Tokyo, University of Tokyo Press, 1979. (In press)

Project No. Z01 HL 03165-01 PA

ANNUAL REPORT OF THE
LABORATORY OF BIOCHEMICAL GENETICS
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1978 through September 30, 1979

Biochemistry of Synaptogenesis. Clonal lines of hybrid cells derived by fusion of neuroblastoma cells with other cell types were shown previously to form synapses with striated muscle cells with high frequency. The formation of synapses between clonal cells of neural origin, such as NBr10A or NG108-15 hybrid cells, and rat striated muscle cells was found to be regulated. Exposure of hybrid cells for 3-7 days to PGE_1, which results in activation of adenylate cyclase, or exposure to various cyclic nucleotide phosphodiesterase inhibitors markedly increases the number of synapses formed. The effects of putative neurotransmitters or hormones on intracellular cyclic AMP or cyclic GMP levels, voltage-sensitive Ca^{2+} channel activity, and acetylcholine secretion were determined. Receptor-mediated increases in intracellular cyclic AMP or cyclic GMP levels had no immediate effect on K^+-dependent $^{45}Ca^{2+}$ uptake by cells or on acetylcholine secretion from cells. However, prolonged exposure of hybrid cells to PGE_1 results both in an increase in cellular cyclic AMP and the gradual acquisition by cells of functional voltage-sensitive Ca^{2+} channels. Concomitantly cells acquire the ability to secrete acetylcholine in response to a depolarizing stimulus and can then form functional synapses with muscle cells. These results show that the acquisition of voltage-sensitive Ca^{2+} channels is regulatedand that this reaction in turn controls the formation of synapses.

D600 inhibits $^{45}Ca^{2+}$ uptake dependent on 80 mM K^+ (IC_{50} = 2 x 10^{-7} M), but has little or no effect on $^{45}Ca^{2+}$ uptake in the presence of 5 mM K^+. $^{45}Ca^{2+}$ uptake also is inhibited by 10 mM La^{3+}, Co^{2+}, Ni^{2+}, Mn^{2+}, Sr^{2+}, or Ba^{2+}, but not by 10 μM tetrodotoxon, 20 mM tetraethylammonium, or 1 mM 3,4-diaminopyridine.

Other cell lines were found that synthesize acetylcholine but do not form synapses with striated muscle cells. Various types of synapse defects were detected; including defects in voltage-sensitive $^{45}Ca^{2+}$ channels, vesicles, and an additional unidentified reaction that is required for acetylcholine secretion.

To identify molecules required for synaptogenesis or communication across the synapse, hybrid cell lines which synthesize mono-specific antibodies were obtained by fusion of clonal myeloma cells with spleen cells immunized against cells from the nervous system. Some of the hybridoma cell lines that were obtained synthesize monospecific antibodies of high titre directed against membrane antigens found on some cells from the nervous system that were not detected with cells from other tissues. One of these cell lines, A2B5, synthesizes antibody directed against an antigen that was shown by indirect immunofluorescence to be associated with plasma membranes of most, or all, neuron cell bodies in chick retina; however, the antigen was not detected on axons or dendrites of neurons, on retina Müller cells, or pigment cells, or on cells from non-neural tissues.

Antigen A2B5 activity is relatively stable at 100°C, is insensitive to trypsin, exhibits the solubility properties of a ganglioside, and is destroyed by neuraminidase. Antibody A2B5 cytotoxicity against retina cells is inhibited by a tetrasialo GQ ganglioside fraction from bovine brain (estimated half-maximal inhibition, 0.2 μM), or N-acetylneuraminic acid (half-maximal inhibition, 5,000

μM), but not by other purified gangliosides tested. These results suggest that the antigen is a GQ ganglioside in plasma membranes of retina neuron cell bodies but not membranes of axons or dendrites.

A solid-phase ^{125}I-Protein A radioassay for anti-cell surface antibodies was devised which employs target cell monolayers cultured on fenestrated polyvinyl chloride 96-well plates ("transfer plates"). The calibrated aperture in the bottom of each well is small enough to retain fluid contents by surface tension during monolayer growth, but also permits fluid to enter the wells when transfer plates are lowered in receptacles containing washing buffer or test sera. To assay for antibodies directed against target cell surface antigens, transfer plates bearing monolayers are inserted into microculture plates with corresponding 96-well geometry, thereby simultaneously sampling 96 wells. This assay allows rapid screening of hundreds of hybrid cell colonies for production of antibodies with desired specificity.

Methyltransferases can be inhibited by S-adenosyl homocysteine or by analogs which either increase S-adenosyl homocysteine levels or inhibit methyltransferases directly such as 3-deazaadenosine(DZA), adenosine-2',3'-diazido-5'-carboxamide (744-99), 5'-deoxy-5'-isobutylthioadenosine(SIBA), and 5'-deoxy-5'-isobutylthio-3-deazaadenosine (DZ-SIBA). In collaboration with P. Chiang and G. Cantoni the effects of these and other compounds on synapses between dissociated chick embryo retina neurons and cultured rat striated muscle cells were investigated. The frequency of spontaneous synaptic responses of muscle cells was markedly reduced by these compounds; half-maximal inhibition was obtained with 1.5×10^{-6} M DZ-SIBA, 1.5×10^{-5} M DZA, 3×10^{-5} M SIBA, or 1×10^{-4} M 744-99. Homocysteine thiolactone, 5-deoxy-adenosine, or tubercidin, which do not increase levels of S-adenosine homocysteine or inhibit methyltransferase activity, do not affect the frequency of spontaneous synaptic responses of muscle cells. These results suggest that a transmethylation reaction may be required for acetylcholine secretion or vesicle cycling in synaptic terminals of neurons.

Regulation of Adenylate Cyclase of Cell Lines From The Nervous System. The inhibition of adenylate cyclase by morphine and the gradual increase in adenylate cyclase activity that results when NG108-15 cells are incubated for 12 or more hours in the presence of morphine was previously proposed as a model for the analgesic action of opiates and for the phenomena of opiate dependence and tolerance. We now find that linoleic acid or serum lipids are required for the morphine-dependent increase in adenylate cyclase activity, but not for inhibition of the enzyme. Similar results were obtained with norepinephrine which activates α-receptors of NG108-15 cells. In this model system, therefore, the inhibition of NG108-15 adenylate cyclase by morphine or norepinephrine can be dissociated from the acquisition of dependence upon opiates or norepinephrine.

Ten μM morphine or norepinephrine completely inhibit the activation of adenylate cyclase by Ca^{2+} ions, but inhibit basal or PGE_1-activated adenylate cyclase by no more than 55 percent in NG108-15 homogenates. The extent of inhibition of adenylate cyclase by morphine or norepinephrine thus is a function of the Ca^{2+} ion concentration and the proportion of adenylate cyclase molecules that are activated by Ca^{2+} ions.

Activation of serotonin receptors of NG108-15 or NCB-20 hybrid cells by serotonin results in cell depolarization, action potentials, and secretion of acetylcholine into the medium. These responses desensitize in less than 15 sec

and are not inhibited or mimicked by LSD. Serotonin also stimulates adenylate cyclase activity of NCB-20 hybrid cells, but the effect of serotonin does not desensitize. Eadie-Scatchard analysis suggests a bimolecular interaction and reveals no evidence of receptor heterogeneity. The Hill interaction coefficient is 1.0, indicating independent, noncooperative reactions. LSD activates adenylate cyclase (K_{act} = 12 nM) and also inhibits the activation of the enzyme by serotonin (K_i = 10 nM). In addition, mianserin and cyproheptadine inhibit serotonin activation of adenylate cyclase (K_i = 43 nM and 95 nM, respectvely) and LSD activation of adenylate cyclase (K_i = 100 nM and 64 nM, respectively). These results show that serotonin and LSD interact during activation of adenylate cyclase.

Binding sites for [^{3}H]LSD were detected in NCB-20 homogenates; the K_{Dapp} was 36 nM, the Hill coefficient was 1.0, and the receptor concentration was 385 fmol/mg of protein. [^{3}H]LSD was displaced by serotonin (K_i = 110-180 nM). These results agree well with those found to be mediated by a serotonin receptor responsive to LSD that mediates activation of adenylate cyclase. Two binding sites for [^{3}H]serotonin were detected in NCB-20 homogenates [K_{Dapp} = 200 nM and 3750 nM] and serotonin-LSD interactions also were detected.

We conclude that NCB-20 hybrid cells possess two species of serotonin receptors, one coupled to activation of adenylate cyclase, the other to cell depolarization and acetylcholine release; that activation of adenylate cyclase does not affect the rate of acetylcholine release, and, conversely, that serotonin-dependent cell depolarization does not affect intracellular levels of cAMP or cGMP in the hybrid cells tested.

Muscarinic Acetylcholine Receptors. [^{3}H]-Quinuclidinyl-benzilate (QNB) was used to study muscarinic acetylcholine receptors in NG108-15 membrane preparations. The apparent dissociation constant of [^{3}H]-QNB is 1 x 10^{-10} M; the average NG108-15 cell possesses 30,000 specific sites for [^{3}H]-QNB. Activation of the receptors with acetylcholine or carbachol results in cell depolarization, a small increase in cellular cGMP, and inhibition of adenylate cyclase. Cell depolarization and rise in cGMP levels desensitize in 30 sec; whereas, the inhibition of adenylate cyclase does not desensitize. Scatchard analysis revealed only one homogeneous class of [^{3}H]-QNB binding sites; however, biphasic rates of [^{3}H]-QNB association with and dissociation from receptors were found. Evidence was obtained for the formation of a dissociable [[^{3}H]-QNB-Receptor] complex which then is converted to a form which dissociates only slowly. Hill coefficients of approximately 1.0 were found for receptor antagonists and approximately 0.5 for receptor activators. A sequential series of reactions were proposed to account for these observations and for the various states of the muscarinic acetylcholine receptor that were detected.

Nicotinic Acetylcholine Receptors. An αBT-horseradish peroxidase conjugate was used to study the distribution of nicotinic acetylcholine receptors in developing chick retina. Incubation of the retina *in vitro* with the conjugate allowed quantitative comparison of developmental stages. αBT-binding synapses were found at the early stages of synapse formation and comprised between 5 and 11% of the inner plexiform layer synapse population during *in ovo* development.

The acetylcholine receptor aggregation factor from neuroblastoma x glioma hybrid cells was partially purified by ion exchange chromatography, gel filtration, and

preparative isoelectric focusing. Factors with similar activity were detected in embryonic brain and cultures of sympathetic ganglion neurons and spinal cord neurons, but not in liver, adult brain, or embryonic glial cell cultures.

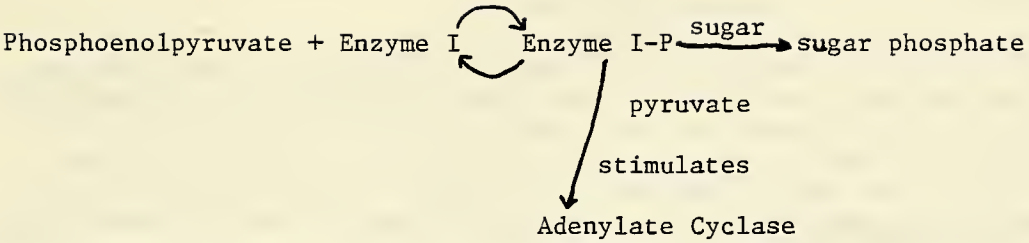
Detergent treatment under appropriate conditions removed most lipid and soluble protein from cultures skeletal muscle cells, but left the cytoskeleton and bound components intact. This extraction was used to distinguish tightly bound and loosely bound populations of acetylcholine receptors, which may be correlated with the degree of receptor aggregation.

Endorphin Synthesis and Secretion. AtT-20 mouse pituitary tumor cells were shown to synthesize and secrete β-endorphin (β-lipotropin$_{61-91}$). The cells contain at least 1 nmole β-endorphin equivalents of opioid peptides per mg cell protein. Analysis of cell extracts by gel filtration and high pressure liquid chromatography indicate that the activity is due to β-endorphin, α-endorphin (β-lipotropin$_{61-76}$), and γ-endorphin (β-lipotropin$_{61-77}$), in the approximate proportions 70%, 24%, and 6%, respectively. Subcellular fractionation indicated that most of the activity is located in the granular fraction. Electron microscopy revealed the presence of osmiophilic granules resembling the secretory granules of corticotrophs of the anterior pituitary. These granules were positive for β-endorphin/β-lipotropin immunoreactivity, when assayed. Thus, AtT-20 tumor cells possess a mechanism similar to that of normal endocrine cells for packaging peptides destined for secretion.

In the absence of serum, basal secretion of β-lipotropin/β-endorphin immunoreactivity is 20-30 pmoles per mg protein per hr and secretion is linear for at least 12 hr. Fifty to 70% of the immunoreactivity secreted is due to β-lipotropin-like peptides and the rest to β-endorphin-like peptides. Thus much β-lipotropin is secreted with further processing.

Secretion is stimulated 5-8 fold by brief exposure of cells to elevated K^+ ion concentration; this stimulation is dependent on Ca^{++} ions. Glucocorticoids, such as dexamethasone, reduce the secretion of β-lipotropin/β-endorphin within 2 hr; for example, secretion is reduced by 33% or 67% after 2 or 8 hrs, respectively. During this time the intracellular content remains the same, however, intracellular content diminishes after 24 hr of treatment. The half-maximally effective dexamethasone concentration is 2 nM. The effect of dexamethasone on secretion is abolished by cycloheximide or actinomycin D which inhibit protein synthesis and RNA synthesis, respectively. This suggests that glucocorticoids act at the transcriptional level to induce the synthesis of protein(s) which inhibit the secretion of ACTH and β-endorphin. Other workers have shown recently that corticotropin/β-lipotropin mRNA is gradually reduced by glucocorticoid treatment for 1-4 days. The present results with AtT-20 cells suggest that glucocorticoids have an earlier different effect on secretion than the slower reduction in mRNA for the prohormone.

Cyclic Nucleotides In E. Coli. Our previous studies led to the development of a model for the regulation of adenylate cyclase involving the phosphoenolpyruvate:sugar phosphotransferase system (PTS). The proposal has been made that Enzyme I of the PTS interacts in a regulatory sense with the catalytic unit of adenylate cyclase:



The phosphoenolpyruvate (PEP)-dependent phosphorylation of Enzyme I is assumed to be associated with a high activity state of adenylate cyclase. The pyruvate or sugar-dependent dephosphorylation of Enzyme I is correlated with a low activity state of adenylate cyclase. Evidence in support of the proposed model involves the observation that Enzyme I mutants have low cAMP levels and that PEP increases cellular cAMP levels and, under certain conditions, activates adenylate cyclase. Kinetic studies indicate that various ligands have opposing effects on adenylate cyclase. While PEP activates the enzyme, either glucose or pyruvate inhibit it. The unique relationships of PEP and Enzyme I to adenylate cyclase activity provide further support for the model outlined above.

The interaction of adenylate cyclase with sugars that are transported by systems other than the PTS also were explored. Sugars such as lactose are transported without modification by a mechanism involving proton cotransport; this mechanism requires a proton motive force across the cell membrane. We have been able to show that uptake of sugars through the lactose transport system results in inhibition of adenylate cyclase activity if the proton symport mechanism is also active. The protonophore carbonyl cyanide *m*-chlorophenylhydrazone also inhibits adenylate cyclase activity. These data suggest that the steady-state electrochemical proton gradient regulates the activity of adenylate cyclase. We propose that sugar-dependent inhibition of adenylate cyclase activity may occur by either of two mechanisms. Sugars transported by the PTS inhibit adenylate cyclase activity by dephosphorylation of a regulatory protein, while sugars transported by the proton motive force system inhibit adenylate cyclase activity as a result of collapse of the proton electrochemical gradient.

Metabolism of Thyrotropin Releasing Hormone. Previously we described an enzyme (pyroglutamate aminopeptidase) in brain extracts that converts TRH to histidyl-prolineamide which spontaneously cyclizes to histidyl-proline diketopiperazine. We also presented evidence of the presence in hypothalamic extracts of an enzyme (TRH deamidase) that converts TRH to pryoglutamyl-histidyl proline. Further studies have led to the isolation from brain of an imidopeptidase for histidyl-prolineamide not previously described. The enzyme was found in extracts of porcine brain acetone powder and purified by conventional column chromatography on DEAE cellulose resulting in the separation of the enzyme from other enzymes that metabolize TRH. The best substrates for the imidopeptidase contain an α-amino group on histidine and a blocked carboxyl group on proline, as is found in histidyl-prolineamide. Other polypeptide hormones were shown to inhibit imidopeptidase activity. Inhibition of the enzyme by adrenocorticotropic hormone (1-24) is noncompetitive. These studies have led us to propose that pituitary hormones may stimulate the production of histidyl-proline diketopiperazine by inhibiting alternate routes of TRH metabolism.

The Biological Activity of Histidyl-Proline Diketopiperazine. Previously we showed that injection of radioactive TRH into rat brain led to the formation of radioactive histidyl-proline diketopiperazine, establishing this compound as a naturally occurring brain peptide. While TRH could antagonize the effects of ethanol in inducing sleep in rats, the dipeptide diketopiperazine was substantially more active than TRH. We therefore suggested that the activity of TRH in antagonizing ethanol narcosis may require its conversion to histidyl-proline diketopiperazine.

We have continued to explore the biological activities of histidyl-proline diketopiperazine and find that it plays a role in thermoregulation and in the regulation of brain cyclic nucleotide levels.

Intraventricular administration of histidyl-proline diketopiperazine to rats produces a dose-dependent hypothermia at 4° or 24°, but not at 31°. At 4° administration of TRH elicits a dose-dependent hypothermia up to 0.1 μmole/Kg which is not evoked at higher doses. At 24°, TRH administration results in no change in temperature, whereas it induces hypothermia at 31°. At 4°, TRH antagonizes and TRH antiserum potentiates the hypothermic effects of histidyl-proline diketopiperazine, suggesting opposing effects of TRH and histidyl-proline diketopiperazine on thermoregulation.

Intraperitoneal administration of thyrotropin releasing hormone (50 μmole/Kg) produced an approximately 2-fold increase in rat brain cGMP concentration within 15 min. Histidyl-proline diketopiperazine produced a similar effect, but the response was faster and shorter-lasting. Intraperitoneal administration of ethanol (1.5 g/Kg) decreased brain cGMP concentration approximately 50% within 10-15 min; thyrotropin releasing hormone or histidyl-proline diketopiperazine, injected 5 min after ethanol, antagonized the ethanol-induced increase in cGMP.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00002-06 LBG

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

Receptor Mediated Regulation of Adenylate Cyclase.

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

PI:	Marshall Nirenberg	Chief, LBG	LBG, NHLBI
OTHERS:	Douglas Wilkening	PRAT Fellow	LBG, NHLBI
	John MacDermot	Visiting Fellow	LBG, NHLBI
	Saburo Ayukawa	Visiting Fellow	LBG, NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section of Molecular Biology

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	3.0	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)

Receptor-mediated activation and inhibition of adenylate cyclase of neuroblastoma x glioma hybrid cells and other cell lines were studied.

1069

Major Findings: The inhibition of adenylate cyclase by morphine and the gradual increase in adenylate cyclase activity that results when NG108-15 cells are incubated for 12 or more hours in the presence of morphine was previously proposed as a model for the analgesic action of opiates and for the phenomena of opiate dependence and tolerance. We now find that linoleic acid or serum lipids are required for the morphine-dependent increase in adenylate cyclase activity, but not for inhibition of the enzyme. Similar results were obtained with norepinephrine which activates α-receptors of NG108-15 cells. In this model system, therefore, the inhibition of NG108-15 adenylate cyclase by morphine or norepinephrine can be dissociated from the acquisition of dependence upon opiates or norepinephrine.

Ten μM morphine or norepinephrine completely inhibit the activation of adenylate cyclase by Ca^{2+} ions, but inhibit basal or PGE_1-activated adenylate cyclase by no more than 55 percent in NG108-15 homogenates. The extent of inhibition of adenylate cyclase by morphine or norepinephrine thus is a function of the Ca^{2+} ion concentration and the proportion of adenylate cyclase molecules that are activated by Ca^{2+} ions.

Activation of serotonin receptors of NG108-15 or NCB-20 hybrid cells by serotonin results in cell depolarization, action potentials, and secretion of acetylcholine into the medium. These responses desensitize in less than 15 sec and are not inhibited or mimicked by LSD. Serotonin also stimulates adenylate cyclase activity of NCB-20 hybrid cells, but this effect of serotonin does not desensitize. Eadie-Scatchard analysis suggests a bimolecular interaction and reveals no evidence of receptor heterogeneity. The Hill interaction coefficient is 1.0, indicating independent, noncooperative reactions. LSD activates adenylate cyclase (K_{act} = 12 nM) and also inhibits the activation of the enzyme by serotonin (K_i - 10 nM). In addition, mianserin and cyproheptadine inhibit serotonin activation of adenylate cyclase (K_i = 43 nM and 95 nM, respectively) and LSD activation of adenylate cyclase (K_i - 100 nM and 64 nM, respectively). These results show that serotonin and LSD interact during activation of adenylate cyclase.

Binding sites for [^{3}H]LSD were detected in NCB-20 homogenates; the K_{Dapp} was 36 nM, the Hill coefficient was 1.0, and the receptor concentration was 385 fmol/mg of protein. [^{3}H]LSD was displaced by serotonin (K_i = 110-180 nM). These results agree well with those found to be mediated by a serotonin receptor responsive to LSD that mediates activation of adenylate cyclase. Two binding sites for [^{3}H]serotonin were detected in NCB-20 homogenates [K_{Dapp} = 200 nM and 3750 nM] and serotonin-LSD interactions also were detected.

We conclude that NCB-20 hybrid cells possess two species of serotonin receptors, one coupled to activation of adenylate cyclase, the other to cell depolarization and acetylcholine release; that activation of adenylate cyclase does not affect the rate of acetylcholine release, and, conversely, that serotonin-dependent cell depolarization does not affect intracellular levels of cAMP or cGMP in the hybrid cells tested.

Significance to Biomedical Research:

The results suggest that fatty acids may be required for cellular acquisition of opiate dependence and tolerance and that the analgesic action of morphine

may be uncoupled from the acquisition of morphine dependence and tolerance.

Publications:

1. McDermot, J., Higashida, H., Wilson, S. P., Matsuzawa, H., Minna, J. and Nirenberg, M. Adenylate Cyclase and Acetylcholine Release Regulated By Separate Serotonin Receptors Of Somatic Cell Hybrids, Proc. Natl. Acad. Sci. USA 76, 1135-1139 (1979).

2. Wilkening, D., and Nirenberg, M. A Lipid Requirement For Acquisition Of Opiate Or Epinephrine Dependence By Neuroblastoma x Glioma Hybrid Cells, J. Neurochem., In Press.

3. Wilkening, D., Sabol, S. L., and Nirenberg, M. Control of Opiate Receptor-Adenylate Cyclase Interactions By Calcium Ions and Guanosine-5'-Triphosphate, Brain Res., In Press.

4. Sabol, S. L., and Nirenberg, M. Regulation of Adenylate Cyclase Of Neuroblastoma x Glioma Hybrid Cells By α-Receptors, I. Inhibition Of Adenylate Cyclase Mediated By α-Receptors, J. Biol. Chem. 254, 1913-1920 (1979).

5. Sabol, S. L., and Nirenberg, M. Regulation Of Adenylate Cyclase Of Neuroblastoma x Glioma Hybrid Cells By α-Adrenergic Receptors. II. Long-lived Increase Of Adenylate Cyclase Activity Mediated By α-Receptors. J. Biol. Chem. 254, 1921-1926 (1979).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00009-05 LBG

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

Cell Recognition and Synapse Formation.

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

PI:	Marshall Nirenberg	Chief, LBG	LBG NHLBI
OTHER:	Andrej Rotter	Visiting Fellow	LBG NHLBI
	Radharaman Ray	Staff Fellow	LBG NHLBI
	Michael Adler	Staff Fellow	LBG NHLBI
	George Eisenbarth	Research Associate	LBG NHLBI
	Frank Walsh	Guest Worker	LBG NHLBI
	Jeffrey Thompson	Staff Fellow	LBG NHLBI

COOPERATING UNITS (if any)

G. Cantoni and P. Chiang, Laboratory of General and Comparative Biochem., NIMH

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section of Molecular Biology

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
7.5	6	1.5

CHECK APPROPRIATE BOX(ES)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Regulatory reactions were identified that turn synapses on or off.

1072

Project Description:

Major Findings: The formation of synapses between clonal cells of neural origin, such as NBr10A or NG108-15 hybrid cells, and rat striated muscle cells was found to be regulated. Exposure of hybrid cells for 3-7 days to PGE_1, which results in activation of adenylate cyclase, or exposure to various cyclic nucleotide phosphodiesterase inhibitors, markedly increases the number of synapses formed. The effects of putative neurotransmitters or hormones on intracellular cyclic AMP or cyclic GMP levels, voltage-sensitive Ca^{2+} channel activity, and acetylcholine secretion were determined. Receptor-mediated increases in intracellular cyclic AMP or cyclic GMP levels had no immediate effect on K^+-dependent $^{45}Ca^{2+}$ uptake by cells or on acetylcholine secretion from cells. However, prolonged exposure of hybrid cells to PGE_1 results both in an increase in cellular cyclic AMP and the gradual acquisition by cells of functional voltage-sensitive Ca^{2+} channels. Concomitantly cells acquire the ability to secrete acetylcholine in response to a depolarizing stimulus and can then form functional synapses with muscle cells.

D600 inhibits $^{45}Ca^{2+}$ uptake dependent on 80 mM K^+ (IC_{50} = 2 x 10^{-7} M), but has little or no effect on $^{45}Ca^{2+}$ uptake in the presence of 5 mM K^+. $^{45}Ca^{2+}$ uptake also is inhibited to 10 mM La^{3+}, Co^{2+}, Ni^{2+}, Mn^{2+}, Sr^{2+}, or Ba^{2+}, but not by 10 µM tetrodotoxin, 20 mM tetraethylammonium, or 1 mM 3,4-diaminopyridine.

Other cell lines were found that synthesize acetylcholine but do not form synapses with striated muscle cells. Various types of synapse defects were detected; including defects in voltage-sensitive $^{45}Ca^{2+}$ channels, vesicles, and an additional unidentified reaction that is required for acetylcholine secretion. These results show that cell lines with or without defects in synapse formation can be generated and that voltage-sensitive Ca^{2+} channel activity can be regulated by a receptor-mediated reaction which is coupled to activation of adenylate cyclase, or by inhibition of cyclic nucleotide phosphodiesterase. Voltage-sensitive Ca^{2+} channel activity increases slowly over a period of days and this reaction is required for stimulus-dependent secretion of transmitter and the formation of functional synapses.

To identify molecules required for synaptogenesis or communication across the synapse, hybrid cell lines which synthesize mono-specific antibodies were obtained by fusion of clonal myeloma cells with spleen cells immunized against cells from the nervous system. Some of the hybridoma cell lines that were obtained synthesize mono-specific antibodies of high titre directed against membrane antigens found on some cells from the nervous system that were not detected with cells from other tissues. One of these cell lines, A2B5, synthesizes antibody directed against an antigen that was shown by indirect immunofluorescence to be associated with plasma membranes of most, or all, neuron cell bodies in chick retina; however, the antigen was not detected on axons or dendrites of neurons, on retina Müller cells, or pigment cells, or on cells from non-neural tissues.

Antigen A2B5 activity is relatively stable at 100°C, is insensitive to trypsin, exhibits the solubility properties of a ganglioside, and is destroyed by

neuraminidase. Antibody A2B5 cytotoxicity against retina cells is inhibited by a tetrasialo GQ ganglioside fraction from bovine brain (estimated half-maximal inhibition, 0.2 μM), or N-acetylneuraminic acid (half-maximal inhibition, 5,000 μM), but not by other purified gangliosides tested. These results suggest that the antigen is a GQ ganglioside in plasma membranes of retina neuron cell bodies but not membranes of axons or dendrites.

A solid-phase ^{125}I-Protein A radioassay for anti-cell surface antibodies was devised which employs target cell monolayers cultured on fenestrated polyvinyl chloride 96-well plates ("transfer plates"). The calibrated aperture in the bottom of each well is small enough to retain fluid contents by surface tension during monolayer growth, but also permits fluid to enter the wells when transfer plates are lowered in receptacles containing washing buffer or test sera. To assay for antibodies directed against target cell surface antigens, transfer plates bearing monolayers are inserted into microculture plates with corresponding 96-well geometry, thereby simultaneously sampling 96 wells. This assay allows rapid screening of hundreds of hybrid cell colonies for production of antibodies with desired specificity.

Methyltransferases can be inhibited by S-adenosyl homocysteine or by analogs which either increase S-adenosyl homocysteine levels or inhibit methyltransferases directly such as 3-deazaadenosine(DZA), adenosine-2',3'-deazido-5'-carboxamide (744-99), 5'-deoxy-5'-isobutylthioadenosine(SIBA), and 5'-deoxy-5'-isobutylthio-3-deazaadenosine(DZ-SIBA). In collaboration with P. Chiang and G. Cantoni the effects of these and other compounds on synapses between dissociated chick embryo retina neurons and cultured rat striated muscle cells were investigated to determine whether inhibition of transmethylation affects synapse formation, acetylcholine release, or muscle responses to acetylcholine mediated by nicotinic acetylcholine receptors. The frequency of spontaneous synaptic responses of muscle cells was markedly reduced by these compounds; half-maximal inhibition was obtained with 1.5×10^{-6} M DZ-SIBA, 1.5×10^{-5} M DZA, 3×10^{-5} M SIBA, or 1×10^{-4} M 744-99. DZ-SIBA reduced the frequency of muscle synaptic responses by 50 percent in 3.5 minutes via a reaction which exhibits first-order kinetics. Homocysteine thiolactone, 5-deoxy-adenosine, or tubercidin, which do not increase levels of S-adenosine homocysteine or inhibit methyltransferase activity, do not affect the frequency of spontaneous synaptic responses of muscle cells. However, homocysteine thiolactone potentiates the inhibition of muscle synaptic responses by DZA by 6-fold. These results suggest that a transmethylation reaction may be required for acetylcholine secretion or vesicle cycling in synaptic terminals of neurons.

Significance to Biomedical Research: Cultured cell systems have been established and used as model systems for biochemical and electrophysiological studies on synapses. A reaction was found that regulates synapse plasticity.

Proposed Course: Current studies focus on determining the reactions which are required for synapse formation and termination and factors regulating these reactions.

1. Nirenberg, M., Wilson, S., Higashida, H., Thompson, J., Eisenbarth, G., Walsh, F., Rotter, A., Kenimer, J., and Sabol, S. Synapse Plasticity. In Pontificiae Academiae Scientarvm Scripta Varia, In Press.

2. Eisenbarth, G. S., Walsh, F. S., and Nirenberg, M. Monoclonal Antibody To A Plasma Membrane Antigen of Neurons, Proc. Natl. Acad. Sci., In Press.

3. Schneider, M. D., and Eisenbarth, G. S. Transfer Plate Radioassay Using Cell Monolayers To Detect Anti-Cell Surface Antibodies Synthesized By Lymphocyte Hybridomas, J. Immunol. Methods, In Press.

4. Eisenbarth, G. S., Ruffalo, R. R., Walsh, F. S., and Nirenberg, M. Lactose Sensitive Lectin Of Chick Retina And Spinal Cord, Biochem. and Biophys. Res. Comm. 83, 1246-1252 (1978).

5. McGee, R., Smith, C., Christian, C., Mata, M., Nelson, P., and Nirenberg, M. A New Method For Measurement Of The Uptake And Release Of Materials From Cultured Cells. Anal. Biochem, In Press.

6. DeMello, F. G., The Ontogeny Of Dopamine-dependent Increase Of Adenosine 3',5'-cyclic Monophosphate In The Chick Retina, J. Neurochem. 31, 1049-1053 (1978).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00012-05 LBG

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (30 characters or less)

Muscarinic Acetylcholine Receptors of Cultured Cell Lines

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

PI:	Marshall Nirenberg	Chief, LBG	LBG NHLBI
OTHER:	William L. Kline	Guest Worker	LBG NHLBI
	Orest Hurko	Staff Associate	LBG NHLBI

COOPERATING UNITS (if any)

None

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NHLBI, NIH, Bethesda, MD 20205

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0.25	0.25	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Studies on muscarinic acetylcholine receptors focus both on ligand-binding and on defining the physical properties of muscarinic receptors.

1076

Project Description:

Major Findings: [^{3}H]-Quinuclidinyl-benzilate (QNB) was used to study muscarinic acetylcholine receptors in NG108-15 membrane preparations. The apparent dissociation constant of [^{3}H]-QNB is 1×10^{-10} M; the average NG108-15 cell possesses 30,000 specific sites for [^{3}H]-QNB. Activation of the receptors with acetylcholine or carbachol results in cell depolarization, a small increase in cellular cGMP, and inhibition of adenylate cyclase. Cell depolarization and rise in cGMP levels desensitize in 30 sec; whereas, the inhibition of adenylate cyclase does not desensitize. Scatchard analysis revealed only one homogeneous class of [^{3}H]-QNB binding sites; however biphasic rates of [^{3}H]-QNB association with and dissociation from receptors were found. Evidence was obtained for the formation of a dissociable [[^{3}H]-QNB·Receptor] complex which then is converted to a form which dissociates only slowly. Hill coefficients of approximately 1.0 were found for receptor antagonists and approximately 0.5 for receptor activators. A sequential series of reactions were proposed to account for these observations and for the various states of the muscarinic acetylcholine receptor that were detected.

Publications:

1. Burgermeister, W., Kline, W.L., Nirenberg, M., and Witkop, B., Mol. Pharm. 14, 751-767 (1978).

2. Hurko, O. Specific [^{3}H]-Quinuclidinyl Benzilate binding activity in digitonin-solubilized preparations for bovine brain. Arch. Biochem. and Biophys. 190, 434-445 (1978).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00017-04 LBG

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

Acetylcholine Receptors

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

PI:	Mathew P. Daniels	Research Biologist	LBG NHLBI
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	C. Christian	Senior Staff Fellow	LDN NICHD
	Z. Vogel	Assistant Professor	Weizmann Institute
	Marshall Nirenberg	Chief, LBG	LBG NHLBI
	Hans Bauer	Visiting Scientist	LDN NICHD
	Joav Prives	Guest Worker	LDN NICHD
	Anne Schaffner	Guest Worker	LBG NHLBI

COOPERATING UNITS (if any)

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Neurobiology Unit, Weizmann Institute of Science

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Our aim is to study the distribution of nicotinic acetylcholine receptors in intact and cultured tissues of the peripheral and central nervous system in relationship to the development and function of synapses. To this purpose histochemical localization of α-bungarotoxin bound to the receptors is used in conjunction with light and electron microscopy. In the past year we have continued our study of the formation of cholinergic synapses in developing chick retina, using an α-bungartoxin-horseradish peroxidase conjugate; we have extended our studies on the control of nicotinic acetylcholine receptor aggregation on cultured skeletal muscle cells by macromolecular factors secreted by neuroblastoma-glioma hybrid cells and embryonic neurons; and we have initiated work on the structural interaction between the cytoskeleton and nicotinic acetylcholine receptors in cultured skeletal muscle cells.

PHS-6040
(Rev. 10-76)

Project Description:

Methods Employed: We have used fluorescence staining of monolayer cultured muscle cells with rhodamine-labeled α-bungarotoxin (αBT) and peroxidase staining of tissues incubated in vitro with peroxidase-labeled αBT. These materials are subsequently examined by light or electron microscopy to visualize and quantitate nicotinic acetylcholine receptor sites (AChR).

Ion exchange chromatography, ultrafiltration, and isoelectric focusing have been used to characterize and purify the AChR aggregation factor. Primary cultures of dissociated embryonic neurons and serial cultures of clonal cell lines have been grown as sources of AChR aggregating factor.

^{125}I-αBT binding, detergent treatment and light and electron microscopy have been used to study AChR-cytoskeleton interactions.

Major Findings: An αBT-horseradish peroxidase conjugate was used to study the distribution of AChR (αBT binding sites) in developing chick retina. Incubation of the retina in vitro with the conjugate allowed quantitative comparison of developmental stages. αBT-binding synapses were found at the early stages of synapse formation and comprised between 5 and 11% of the inner plexiform layer synapse population during in ovo development.

The AChR aggregation factor from neuroblastoma x glioma hybrid cells was partially purified by ion exchange chromatography, gel filtration, and preparative isoelectric focusing. Factors with similar activity were detected in embryonic brain and cultures of sympathetic ganglion neurons and spinal cord neurons, but not in liver, adult brain or embryonic glial cell cultures.

Detergent treatment under appropriate conditions removed most lipid and soluble protein from cultured skeletal muscle cells, but left the cytoskeleton and bound components intact. This extraction was used to distinguish tightly bound and loosely bound populations of AChR, which may be correlated with the degree of receptor aggregation.

Significance to Biomedical Research: Knowledge of the ultrastructural distribution of acetylcholine receptors is of clear importance in any attempt to understand the role of neurotransmitters and their receptors in the function and development of the nervous system.

The results obtained with developing chick retina represent the beginning of an understanding of the role of neurotransmitter receptors in the formation and maturation of chemical synapses, as seen on the ultrastructural level.

The cultured muscle studies may lead to a better understanding of the mechanism whereby neurons control the distribution of receptors on muscle cells and on other neurons.

Proposed Course of Research:
(1) We have developed a monolayer culture system for physiological and histochemical observation of rabbit retina neurons, which we hope to exploit to learn more about the relationships between αBT binding sites and AChR in central neurons.

(2) We will continue the biochemical characterization of the AChR aggregation factor, adding immunochemical techniques to the array. We will also continue to probe the cellular specificity of factor formation and target receptor specificity of the factor.

(3) We will pursue the study of AChR-cytoskeletal interactions with biochemical and morphological techniques.

Publications:

1) Christian, C.N., Daniels, M.P., Sugiyama, H., Vogel, Z., Jacques, L., and Nelson, G.: A factor from neurons increases the number of acetylcholine receptor aggregates on cultured muscle cells. Proc. Natl. Acad. Sci. USA 75: 4011-4015 (1978)

2) Vogel, Z., Towbin, M., and Daniels, M.P.: α-Bungarotoxin-horseradish peroxidase conjugate: Preparation, properties and utilization for the histochemical detection of receptors to acetylcholine. J. Histochem. Cytochem. 27: 846-851, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL00018-02 LBG

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

Regulation of synthesis and secretion of endorphins by pituitary tumor cells

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

PI:	Steven L. Sabol	Medical Officer	LBG NHLBI
OTHERS:	Mathew Daniels	Research Biologist	LBG NHLBI
	Alice Ling	Staff Biologist	LBG NHLBI
	Michael Adler	Staff Fellow	LBG NHLBI

COOPERATING UNITS (if any)

None.

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1.3	1.3	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

AtT-20 mouse pituitary tumor cells synthesize and secrete corticotropin, β-lipotropin, and several peptides with opiate activity; β-, α-, and γ-endorphins. Aspects of the regulation of biosynthesis and secretion of these peptide hormones are being studied in this clonal cell line, which is a useful model system for the corticotropin-β-lipotropin producing cells of the anterior pituitary gland. Studies during the past year have concentrated on the secretion of β-lipotropin and β-endorphin by the cells. These aspects include (1) the packaging of these peptides into secretory granules, studied by immunocyotchemistry, (2) the inhibition of the secretion of peptide hormones by glucocorticoids, acting through glucocorticoid receptors, and (3) studies on the electrical excitability of AtT-20 cells and its enhancement by dibutyryl cyclic AMP.

1051

Project Description

Objectives: Peptides with opiate activity, termed endorphins, which are synthesized in the nervous system, intestinal tract, and pituitary gland, are endogenous ligands of the opiate receptor. AtT-20 mouse pituitary tumor cells were found by us to synthesize and secrete β-endorphin (β-lipotropin$_{61-91}$). Other workers found that in AtT-20 and pituitary cells, corticotropin and β-lipotropin are synthesized from a common prohormone; β-endorphin is subsequently cleaved from β-lipotropin. Studies in our laboratory have attempted to determine whether the AtT-20 system is a valid model for studying the regulation of secretion of corticotropin and endorphin. Since corticotrophs constitute a small fraction of pituitary cells, a clonal cell line would be an ideal system in which to study the molecular basis of regulation of secretion by corticotropin-releasing-factor and glucocorticoids.

Methods Employed: Total endorphin activity of cell extracts was assayed in a novel way by opiate-receptor mediated inhibition of adenylate cyclase activity of NG 108-15 neuroblastoma x glioma hybrid cell homogenates. β-endorphin + β-lipotropin immunoreactivity was measured by a radioimmunoassay 100 times more sensitive than the adenylate cyclase assay. Secretion studies were carried out on the AtT-20 variant clone D16, or on a possibly more homogeneous subclone, D16/16, which was derived by S. Sabol.

Major Findings: AtT-20 cells contain at least 1 nmole β-endorphin equivalents of opioid peptides per mg cell protein. Analysis of cell extracts by gel filtration and high pressure liquid chromatography indicated that this activity is due to β-endorphin, α-endorphin (β-lipotropin$_{61-76}$), and γ-endorphin (β-lipotropin$_{61-77}$) in the approximate proportions 70%, 24%, and 6%, respectively. Subcellular fractionation studies indicated that most of the activity is located in the granular fraction. Electron microscopy revealed the presence of osmiophilic granules resembling the secretory granules of corticotrophs of the anterior pituitary. These granules were positive for β-endorphin/β-lipotropin immunoreactivity when studied by M. Daniels and A. Ling with the peroxidase-antiperoxidase staining method for ultra-thin sections. Thus, AtT-20 tumor cells possess a mechanism similar or identical to that of normal endocrine cells for packaging peptides destined for secretion.

The AtT-20 variant is more satisfactory than the original AtT-20 line for secretory studies because the former clone releases less protease activity than does the latter clone. In the presence of culture medium without serum, basal secretion of β-lipotropin/β-endorphin immunoreactivity is 20-30 pmoles per mg protein per hr and is linear for at least 12 hr. Gel filtration analysis indicated that 50-70% of the immunoreactivity is due to β-lipotropin-like peptides and the rest to β-endorphin-like peptides. Thus much β-lipotropin is secreted without further processing.

Secretion is stimulated 5-8 fold by brief exposure of cells to elevated K^+ ion concentration, presumably by depolarization of the plasma membrane; this stimulation has an absolute dependence on Ca^{++} ions in the extracellular medium. Glucocorticoids, such as dexamethasone, reduce the secretion of β-lipotropin/β-endorphin within 2 hr of exposure; for example, secretion is reduced by 33%

or 67% after 2 or 8 hrs, respectively. During this time the intracellular content remains the same, however, intracellular content diminishes after 24 hr of treatment. Exposure to dexamethasone for 2 or more hr also results in a reduction (35%) in the K^+-stimulated secretion of β-endorphin immunoreactivity. The half-maximally effective dexamethasone concentration eliciting this response is 2 nM, which is similar to that eliciting a half-maximal decrease of intracellular endorphin levels over a 2-4 day period. This low concentration is consistent with the proposal that glucocorticoid receptors mediate the effect of dexamethasone. The effect of dexamethasone on secretion is abolished by addition of cycloheximide or Actinomycin D to inhibit protein synthesis or RNA synthesis, respectively. This suggests that glucocorticoids act at the transcriptional level to induce the synthesis of protein(s) which somehow inhibit the secretion of ACTH and β-endorphin. Other workers have shown recently that corticotropin/β-lipotropin mRNA is gradually reduced by glucocorticoid treatment for 1-4 days. The present results with AtT-20 cells suggest that glucocorticoids have an earlier effect on secretion which may have a different biochemical basis from the more slowly developing reduction of mRNA levels.

The electrical excitability of AtT-20/D16 cells was investigated by microelectrode recording techniques by M. Adler and H. Higashida. A small fraction of AtT-20 cells exhibited evoked action potentials which consist of sodium and calcium currents. Upon treatment with 1 mM dibutyryl cyclic AMP for 1-4 weeks, the cells become highly excitable and exhibit spontaneous as well as evoked action potentials; in fact, such cells are among the most excitable cultured clonal cells known. When sodium and potassium conductances are blocked, calcium spikes are readily detected. Electrically excitable endocrine cells thought to be derived from the neural crest have been found by others in the pituitary and pancreatic islets; however, electrical excitability of corticotrophs has not been so far reported.

Significance to Biomedical Research: The results indicate that AtT-20 cells are an excellent system to study the regulation of biosynthesis and secretion of the corticotropin/β-endorphin family of peptides. In particular, the molecular mechanisms of the feedback regulation of glucocorticoids on the coordinate release of these peptides are amenable to study in a homogeneous clonal cell system that would appear to offer some advantages over systems composed of all types of pituitary cells. Knowledge of these mechanisms is important to understand the normal function of the pituitary/adrenal axis and its derangements, such as Cushing's disease. The electrical excitability of AtT-20/D16v cells is consistent with the idea that certain endocrine cells, including corticotrophs, may have features in common with neurons, such as a common embryological origin and similar mechanisms of stimulus-secretion coupling.

Proposed Course:

1. A search is being made for factors which potentiate the effect of glucocorticoids in inhibiting the secretion of β-lipotropin/β-endorphin.

2. In collaboration with M. Daniels and A. Ling, immunocytochemistry at the electron microscopic level will be repeated to localize both β-endorphin and corticotropin using a recently described gold colloid-protein A labelling tech-

3. In collaboration with M. Adler, the ionic mechanisms of action potential generation in AtT-20/D16 cells will be characterized and correlated with secretory activity.

4. The project will be expanded to study the biosynthesis of other neural peptides, where possible in clonal cell lines.

Publications:

1. Sabol, S.L., Ling, A., and Daniels, M.P. Endorphins of clonal pituitary tumor cells: synthesis, storage, secretion, and regulation by glucocorticoids. In Hormones and Cell Culture (Cold Spring Harbor Conferences on Cell Proliferation, Vol. 6) (Sato, G., and Ross, R., eds.). Cold Spring Harbor Laboratory, New York, in press, August 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00151-09 LBG

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
The Biology of Cyclic Nucleotides in E. coli

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

PI:	Alan Peterkofsky	Research Chemist Chief, Section on Macromolecules	LBG, NHLBI
OTHER:	Robert Puskas	Staff Fellow	LBG, NHLBI

COOPERATING UNITS (if any)

None.

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

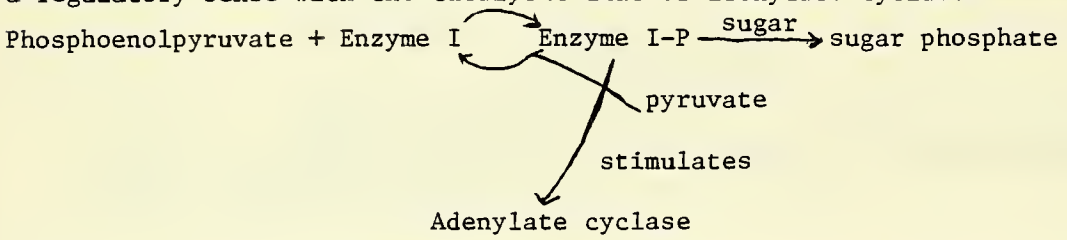
The long-range goal of this project is to delineate the mechanisms involved in the regulation of the synthesis and metabolism of cyclic nucleotides in E. coli. Our recent findings showed that the system for sugar transport referred to as the phosphoenolpyruvate:sugar phosphotransferase system (PTS) plays a role in the regulation of adenylate cyclase. We proposed that Enzyme I of the PTS serves as a regulator of adenylate cyclase. Support for the model that the phosphorylated state of Enzyme I is associated with a condition of stimulation of adenylate cyclase activity comes from studies showing that glucose or pyruvate inhibit adenylate cyclase activity, while phosphoenolpyruvate stimulates the activity. We have also begun to explore the mechanism by which sugars transported by a mechanism involving protonmotive force inhibit adenylate cyclase. Lactose transport is coupled to the inhibition of adenylate cyclase activity only if the proton symport mechanism is also active. Our current hypothesis is that adenylate cyclase can be inhibited by two mechanisms. Transport of PTS sugars decreases the steady-state level of the phospho-form of the PTS protein Enzyme I; transport of sugars linked to a proton symport mechanism decreases the proton gradient. Either of these effects produces inhibition of adenylate cyclase activity.

1085

Project Description:

Major Findings:

Our previous studies have led to the development of a model for the regulation of adenylate cyclase involving the phosphoenolpyruvate:sugar phosphotransferase system (PTS). The proposal has been made that Enzyme I of the PTS interacts in a regulatory sense with the catalytic unit of adenylate cyclase:



The phosphoenolpyruvate (PEP)-dependent phosphorylation of Enzyme I is assumed to be associated with a high activity state of adenylate cyclase. The pyruvate or sugar-dependent dephosphorylation of Enzyme I is correlated with a low activity state of adenylate cyclase. Evidence in support of the proposed model involves the observation that Enzyme I mutants have low cAMP levels and that PEP increases cellular cAMP levels and, under certain conditions, activates adenylate cyclase. Kinetic studies indicate that various ligands have opposing effects on adenylate cyclase. While PEP activates the enzyme, either glucose or pyruvate inhibit it. The unique relationships of PEP and Enzyme I to adenylate cyclase activity provide further support for the model outlined above.

Another study initiated during this year was designed to explore the interaction of adenylate cyclase with sugars that are transported by systems other than the PTS. Sugars such as lactose are transported without modification by a mechanism involving proton cotransport; this mechanism requires a proton motive force across the cell membrane. We have been able to show that uptake of sugars through the lactose transport system results in inhibition of adenylate cyclase activity if the proton symport mechanism is also active. The protonophore carbonyl cyanide *m*-chlorophenylhydrazone also inhibits adenylate cyclase activity. These data suggest that the steady-state electrochemical proton gradient regulates the activity of adenylate cyclase. We propose that sugar-dependent inhibition of adenylate cyclase activity may occur by either of two mechanisms. Sugars transported by the PTS inhibit adenylate cyclase activity by dephosphorylation of a regulatory protein, while sugars transported by the proton motive force system inhibit adenylate cyclase activity as a result of collapse of the proton electrochemical gradient.

Proposed Course of Research:

Our intention during the coming year is to gather more data relating to the conditions for regulating adenylate cyclase by sugar substrates of the lactose transport system. We hope that such studies will provide a basis for clarifying the complexities of the mechanism by which adenylate cyclase in E. coli is controlled.

Publications:

1) Peterkofsky, A., and Gazdar, C. The Escherichia coli adenylate cyclase complex: Activation by phosphoenolpyruvate. J. Supramolecular Structure 9: 219-230, 1978.

2) Peterkofsky, A., and Gazdar, C. Escherichia coli adenylate cyclase complex: Regulation by the proton electrochemical gradient. Proc. Nat. Acad. Sci. USA 76: 1099-1103, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00152-05 LBG

PERIOD COVERED
October 1, 1978 - September 30, 1979

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
OTHERS:	Chandan Prasad	Staff Fellow	LBG, NHLBI
	Tadashi Yanagisawa	Visiting Fellow	LBG, NHLBI
	Fiorenzo Battaini	Visiting Associate	LBG, NHLBI

COOPERATING UNITS (if any)

None.

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

We have continued our studies on the biology of the hypothalamic releasing factor, thyrotropin releasing hormone. We have isolated and characterized an enzyme from porcine brain extracts that has an imidopeptidase action on histidylprolineamide. The isolated enzyme is inhibited noncompetitively by a variety of polypeptide hormones, suggesting that the metabolism of thyrotropin releasing hormone may be regulated by pituitary hormones. We have accumulated increasing evidence that a metabolite of thyrotropin releasing hormone, histidyl-proline diketopiperazine, is a biologically active compound. In addition to antagonizing ethanol-induced sleep in rats, the diketopiperazine produces hypothermia in rats and transiently elevates the level of cGMP in rat brain. We conclude that thyrotropin releasing hormone is an active principle in its own right, but that the enzymatic conversion to histidyl-proline diketopiperazine plays an important role in modulating the effects of the hormone.

1088

Project Description:

Major Findings:

The studies we reported last year dealt with two aspects of the biology of thyrotropin releasing hormone (TRH): (a) enzymes involved in the metabolism of the peptide and (b) the biological activity of histidyl-proline diketopiperazine, which is formed as one of the metabolites of TRH.

(A) Enzymes involved in the metabolism of thyrotropin releasing hormone

In our previous studies, we have described an enzyme (pyroglutamate aminopeptidase) in brain extracts that converts TRH to histidyl-prolineamide which spontaneously cyclizes to histidyl-proline diketopiperazine. We also presented evidence for the presence in hypothalamic extracts of an enzyme (TRH deamidase) that converts TRH to pyroglutamyl-histidyl proline.

Our continuing studies in this area have now led to the isolation from brain extracts of an enzyme, not previously described, which is an imidopeptidase for histidylprolineamide. The enzyme was found in extracts of porcine brain acetone powder and purified by conventional column chromatography on DEAE-cellulose resulting in an isolation from the other enzymes that metabolize TRH. A study of the specificity of the imidopeptidase indicates that the best substrates for the enzyme contain an α-amino group on histidine and a blocked carboxyl group on proline, as is found in histidyl-prolineamide. A survey of a variety of polypeptide hormones indicated that many of them inhibit the imidopeptidase activity. A kinetic study of the inhibition of the enzyme by adrenocorticotropic hormone (1-24) shows that the inhibition by polypeptide hormones is noncompetitive. These studies have led us to propose that pituitary hormones may stimulate the production of histidyl-proline diketopiperazine by inhibiting alternate routes of TRH metabolism.

(B) The biological activity of histidyl-proline diketopiperazine

Last year's report described our findings that injection of radioactive TRH into rat brain led to the formation of radioactive histidyl-proline diketopiperazine, establishing this compound as a naturally occurring brain peptide. We carried out studies showing that, while TRH could antagonize the effects of ethanol in inducing sleep in rats, the dipeptide diketopiperazine was substantially more active than TRH. We therefore suggested that the activity of TRH in antagonizing ethanol narcosis may require its conversion to histidyl-proline diketopiperazine.

We have continued to explore the biological activities of histidyl-proline diketopiperazine and find that it plays a role in thermoregulation and in the regulation of brain cyclic nucleotide levels.

Intraventricular administration of histidyl-proline diketopiperazine to rats produces a dose-dependent hypothermia at 4° or 24°, but not at 31°. At 4°, administration of TRH elicits a dose-dependent hypothermia up to 0.1 μmole/Kg which is not evoked at higher doses. At 24°, TRH administration results in no

change in core temperature, whereas it induces hyperthermia at 31°. At 4°, TRH antagonizes and TRH antiserum potentiates the hypothermic effects of histidyl-proline diketopiperazine, suggesting opposing effects of TRH and histidyl-proline diketopiperazine on thermoregulation.

Intraperitoneal administration of thyrotropin releasing hormone (50 μmole/Kg) produced an approximately 2-fold increase in rat brain cGMP concentration within 15 min. Histidyl-proline diketopiperazine produced a similar effect, but the response was faster and shorter-lasting. Intraperitoneal administration of ethanol (1.5 g/Kg) decreased brain cGMP concentration approximately 50% within 10-15 min; thyrotropin releasing hormone or histidyl-proline diketopiperazine, injected 5 min after ethanol, antagonized the ethanol-induced decrease in cGMP. Antagonism of the ethanol-induced decrease in the cGMP level required 10 μmol/Kg of thyrotropin releasing hormone but was observed with 5 μmol/Kg of histidyl-proline diketopiperazine. These data suggest that the metabolic conversion of thyrotropin releasing hormone to histidyl-proline diketopiperazine might explain the previous observation that thyrotropin releasing hormone elevated the level of brain cGMP and antagonized the ethanol-induced decrease in brain cGMP concentration.

Proposed Course of Research: We have now observed that histidyl-proline diketopiperazine is active in the antagonism of ethanol narcosis, in the regulation of body temperature and in the control of cyclic GMP concentration in brain, thereby establishing this peptide as a significant physiological modulator. In the coming year, we hope to quantitate the amount and distribution of histidyl-proline diketopiperazine in brain and make some attempts to understand its mechanism of action.

Publications:

1) Prasad, C., Matsui, T., Williams, J., and Peterkofsky, A.: Thermoregulation in rats: Opposing effects of thyrotropin releasing hormone and its metabolite histidyl-proline diketopiperazine. Biochem. Biophys. Res. Communs. 85: 1582-1587, 1978.

2) Yanagisawa, T., Prasad, C., Williams, J., and Peterkofsky, A.: Antagonism of ethanol-induced decrease in rat brain cGMP concentration by histidyl-proline diketopiperazine, a thyrotropin releasing hormone metabolite. Biochem. Biophys. Res. Communs. 86: 1146-1153, 1979.

3) Matsui, T., Prasad, C., and Peterkofsky, A.: Metabolism of Thyrotropin releasing hormone in brain extracts: Isolation and characterization of an imidopeptidase for histidyl prolineamide. J. Biol. Chem. 254: 2439-2445, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00153-01 LBG

PERIOD COVERED
March 1, 1979 - September 30, 1979

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:	Daphna Atlas	Visiting Scientist	LBC NIAMDD
	Steven L. Sabol	Medical Officer	LBG NHLBI

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Laboratory of Bioorganic Chemistry, NIAMDD

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section on Molecular Biology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	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)

Neuroblastoma x glioma NG108-15 hybrid cells possess α-adrenergic receptors that, when activated by an α-adrenergic agonist such as norepinephrine, mediate inhibition of adenylate cyclase. Affinity labels are being designed to determine the macromolecule(s) constituting the NG108-15 α-receptor. A number of chemically reactive derivatives of α-adrenergic agonist and antagonist compounds are being synthesized and tested for their ability to bind specifically to and react covalently with α-receptors. In addition, novel α-receptor ligands that can be iodinated to high specific radioactivity are being synthesized and tested for interaction with NG108-15 and brain α-receptors.

1091

Project Description

Objective: Clonal NG108-15 neuroblastoma x glioma hybrid cells constitute a useful system of homogeneous cells in which to study α-adrenergic receptors and their biochemical actions. Previously we found that activation of NG108-15 α-receptors results in inhibition of basal and prostaglandin E_1-stimulated adenylate cyclase activity and a consequent reduction of intracellular cyclic AMP levels (ref. 1). The pharmacological specificity of this effect resembles that of α_2-receptors more than that of α_1-receptors. Alpha-receptors in NG108-15 membranes have been found by S. Ayukawa and M. Nirenberg using direct binding techniques employing [^{3}H]dihydroergocryptine as a ligand. We are currently developing biochemical techniques to attempt to identify and possibly isolate the α-receptor molecule.

Major Findings: Previous studies (ref. 1) showed that with respect to α-receptor mediated inhibition of adenylate cyclase activity, clonidine is a potent mixed agonist-antagonist (apparent K_d 0.1 μM) and yohimbine is a potent antagonist (apparent K_d 0.07 μM). Both compounds have higher affinity for α_2 than for α_1 receptors in other tissues. Thus, chemically reactive derivatives of clonidine and yohimbine, as well as of the classical α-blocker phentolamine, and of haloperidol (an α_1 and dopamine receptor antagonist), are being synthesized and evaluated for suitability as affinity ligands. These include bromoacetyl-p-aminoclonidine, p-fluorosulfonyl-yohimbine, bromoacetyl-phentolamine, and bromoacetyl-aminohaloperidol. The bromoacetyl or fluorosulfonyl groups will react with sulfhydryl and amino groups near the ligand binding site of the receptor molecule. Proteins labeled in a specific manner by a radioactive affinity label can then be analyzed by gel electrophoresis and other techniques. Data on the reactivity of these compounds and their effects on the α-receptor-adenylate cyclase system are currently being gathered.

In addition several novel α-ligands have been synthesized and tested in the NG108-15 adenylate cyclase assay. These include the following.

		K_d (apparent)
I	amino-clonidine (mixed agonist-antagonist)	0.05 μM
II	p-OH-phenacetyl-aminoclonidine (agonist)	0.6 μM
III	p-OH-phenacetyl-aminohaloperidol	0.6 μM

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. However, this receptor is generally understood only in pharmacological terms and is just beginning to be studied biochemically. In addition, several pharmacological subclasses of α-receptors, such as α_1 and α_2 must be biochemically differentiated, if possible. The use of affinity labels, particularly those preferring α_1 or α_2 receptors, should enable the identification of the ligand binding molecules constituting the receptor(s). In addition, the NG108-15 system is an appropriate system to study α_2-receptor-adenylate cyclase interaction.

Compound I above is a relatively potent α-receptor ligand which can be derivatized with a variety of useful chemical groups to produce α-receptor probes for various analytical methodologies. Compound II can be iodinated to very high specific radioactivity, although its biological potency appears relatively low in NG108-15 cells.

Proposed Course: Affinity labels which appear most promising will be utilized to label the NG108-15 α-receptor(s). The hydrodynamic properties, including molecular weight of the solubilized receptor could then be studied in the crude state. The affinities of the various α-ligands described will also be assessed in the brain membrane system, which possesses several types of α-receptors possibly different from those of NG108-15 cells.

Publications:

1. Sabol, S.L., and Nirenberg, M. Regulation of adenylate cyclase of neuroblastoma x glioma hybrid cells by α-adrenergic receptors. I. Inhibition of adenylate cyclase mediated by α-receptors. J. Biol. Chem. 254, 1913-1920 (1979).

2. Sabol, S.L., and Nirenberg, M. Regulation of adenylate cyclase of neuroblastoma x glioma hybrid cells by α-adrenergic receptors. II. Long-lived increase of adenylate cyclase activity mediated by α-receptors. J. Biol. Chem. 254, 1921-1926 (1979).

Annual Report of the
Clinical Hematology Branch
National Heart, Lung, and Blood Institute
October 1, 1978 to September 30, 1979

The research efforts of this Branch are directed toward understanding the underlying causes and potential treatment of the major red cell disorders. Red cell diseases which produce significant morbidity and mortality include thalassemia, severe hemoglobinopathies of which sickle cell anemia is the most common, and the various syndromes of bone marrow failure. Thus various projects in this Branch are directed toward analysis of normal red cell differentiation and maturation, regulation of synthesis of various specific hemoglobins (e.g., fetal and adult hemoglobin in man), and the function of hemoglobin in the transfer of oxygen to tissues. The clinical effects of various anemias as they pertain to organ function are under investigation. Many patients with chronic anemia require regular blood transfusions and ultimately develop pathological iron overload. Efforts are directed towards defining the extent of organ dysfunction due to iron and the potential benefit of iron chelation in these patients.

Hemoglobin is a tetrameric molecule composed of four polypeptides; two are alpha globin and two are either beta or gamma globin. The human beta and gamma globins are encoded by closely linked genes on chromosome 11, whereas the alpha globin gene(s) lie on chromosome 16. Expression of gamma globin in fetal life results in the production of fetal hemoglobin (Hb F - $\alpha_2\gamma_2$) whereas expression of the related and closely linked beta globin gene in postnatal life results in the production of adult hemoglobin (Hb A - $\alpha_2\beta_2$) A major focus of the research work in this Branch is an attempt to elucidate the manner in which this developmental switch in hemoglobin synthesis is regulated and to attempt to devise some means to increase fetal hemoglobin synthesis in patients with severe disorders of hemoglobin structure or production.

The experimental model which we have chosen is the sheep for this species exhibits a fetal to adult hemoglobin switch that is quite analogous to that which occurs in man. Furthermore, certain sheep have an additional beta globin gene which is specifically activated during anemia, apparently by a direct action of the hormone, erythropoietin. Thus, the switch to Hb C production is a readily maniputable phenomenon which can be approached experimentally. Our analysis of hemoglobin switching focuses on the sheep model although immediate extension of important experimental approaches to humans is made whenever possible. Our work is organized along two lines; molecular analysis of globin gene structure and expression in developing erythroblasts and cellular analysis of commitment to express particular globin genes which occurs in early erythroid stem cells.

Analysis of globin gene structure and organization has been greatly facilitated by recombinant DNA and molecular cloning technology. During the past year, we have obtained several recombinants which include sheep globin genes (Individual Project Report: " Regulation of the Sheep Globin Genes"). These include the gamma globin gene expressed during fetal life, the β^A globin gene expressed during adult life, and an embryonic β-like gene expressed during very early fetal development. Several other recombinants containing globin

gene segments have been identified and are in various stages of characterization. The goal of this avenue of investigation is to build up a general map of the organization of the globin genes, to define the distances between the genes, and to determine their general structure. To date we have learned that the γ, β^A and embryonic globin genes contain a small and large intervening sequence of DNA which is transcribed into RNA but is not represented in the mature globin mRNA in the cell cytoplasm. Another immediate goal is to obtain the sequence of the DNA in regions which are potentially critically important. These would include the points at which RNA synthesis begins and ends. We will look for similarities or differences among the several genes which might provide clues for subsequent experimentation directed at understanding the differential expression of these genes during development and erythropoietic stress.

Current evidence suggests that chromatin structure restricts the total number of genes expressed in differentiated cells. We have also directed our efforts toward defining the fine structure of the individual globin genes (Individual project report: "Structure of the globin genes in chromatin"). Pancreatic DNAse I is used as a probe for globin gene structure since transcriptionally active genes in isolated nuclei are exquisitely sensitive to this enzyme. Earlier studies had shown that the γ globin gene but not the beta globin genes were DNAse I sensitive in nuclei from fetal erythroid cells. Coincident with the developmental switch to adult hemoglobin synthesis, the β globin genes become transcriptionally active as defined by their sensitivity to DNAse I, but the γ globin genes also remain in the transcriptionally active conformation. These studies have defined two levels of regulation, one occurring at the level of chromatin structure in fetal cells and a second, apparently operative in adult cells, whereby the rate of transcription of potentially active genes is modulated, leading to differential accumulation of specific mRNAs. The availability of the cloned sheep globin genes provides DNA fragments which can be used as probes in an attempt to define the chromatin structure of the DNA sequences which surround these genes. We anticipate that it will be possible to define the size of the transcriptional unit and to learn whether all of the globin genes are included in a single transcriptional unit in chromatin or whether there are individual transcription units for each of the genes. Our ultimate goal is to use this information to attempt to find those factors important for determining chromatin structure by devising an in vitro reconstitution system.

The thalassemias are congenital anemias characterized by deficient synthesis of one of the globin components of the hemoglobin molecule. In beta thalassemia, a small amount of beta globin may be produced or alternatively - beta globin synthesis may be completely absent. Thus these disorders represent examples of a regulatory mutation. Our analysis is focused on identifying the molecular lesions in these disorders with the anticipation that insights into normal gene regulation may be achieved (Individual Project Report: "Molecular Defect in Beta Thalassemia"). The quantitative deficiency in beta globin mRNA which accounts for the deficient globin synthesis in most patients could arise because of impaired transcription of the gene, defective processing of the immediate product of transcription, or lowered stability of the final globin mRNA. The molecular cloning technology is being used by us in collaboration with the Laboratory of Molecular Hematology to obtain beta

globin genes which are affected by a thalassemia mutation in pure form and in adequate quantities to be used for structural analysis. Furthermore, using a segment of the cloned human genes as probes, we have devised an assay for the precursor to cytoplasmic beta globin mRNA. By comparing the ratio of precursor to final product, we hope to identify any patients who have a mutation which affects processing. Finally, the stability of beta globin mRNA in the cytoplasm is being measured directly by incubation of erythroid cells in [^{3}H]uridine, a precursor to RNA. After the RNA is labelled, prolonged incubation of the cells in vitro allows the stability of β globin mRNAs to be estimated.

During the past year the major focus of the molecular aspects of our work has been directed toward isolating sheep and human globin genes. These will be used in structural studies but it is also important to devise means to test their function in a defined environment. One means of achieving this goal is to introduce DNA into tissue culture cells. Direct transformation of mouse fibroblasts and identification of stable transformants may be accomplished if a selectable marker is available (Individual Project Report: "Transformation of Mammalian Cells"). The thymidine kinase gene from Herpes simplex virus provides such a marker. Using mouse fibroblasts deficient in thymidine kinase, one can transfer the viral gene in by incubating the cells in purified DNA. By mixing the thymidine kinase gene with another sample of DNA, for example the cloned DNA fragment which includes the human beta globin gene, co-transformation occurs. Integration of the thymidine kinase gene may often be accompanied by simultaneous integration of the human beta globin gene. In our initial transformation experiments, we have obtained 11 cell clones which appear to have the viral gene integrated because they grow in selective media. These are being tested to determine whether they also contain the human beta globin gene. Further experiments along these lines will include transformation with β globin genes affected by a thalassemia mutation and also the various sheep globin genes. The behavior of the globin genes with regard to production of an RNA product and synthesis of the various globins will be compared. Furthermore, it may be possible to fuse the transformed fibroblasts to thymidine kinase deficient mouse erythroleukemia cells thereby transferring the viral gene along with the various globin genes into an erythroid cell environment.

The second general approach to understanding the regulation of globin genes utilizes culture techniques to examine erythroid stem cells. These cells, present in small numbers, cannot be defined morphologically but can be recognized by the development of progeny colonies in semisolid media. The more primitive of these stem cells (BFU-E) have high proliferative potential and give rise to very large colonies in vitro whereas the more mature stem cells (CFU-E) with a lower proliferative potential gives rise to smaller colonies. The erythropoietin induced synthesis of Hb C in sheep cells has been explored extensively using this cell culture methodology (Individual Project: "Cellular Analysis of Hemoglobin Switching in Sheep"). We have learned that erythroid stem cells are committed, early in their development, to give rise to progeny erythroblasts which then express the pattern of hemoglobin synthesis determined at the commitment stage. Current evidence suggests that the concentration of erythropoietin to which a stem cell is exposed at a critical phase in its development determines the relative proportions of β^A and β^C glo-

bin synthesis in its progeny erythroblasts. The molecular means by which erythropoietin exerts this action is unknown although we have learned that erythropoietin accelerates the rate of cell division and therefore presumably changes the duration of various parts of the cell cycle during differentiation and maturation of the stem cells into erythroblasts.

Even the earliest stem cells that we are currently able to assay in our in vitro system are already committed with regard to fetal or adult hemoglobin synthesis. Thus, erythroid colonies derived from stem cells in fetal tissue make fetal hemoglobin whereas erythroid colonies derived from stem cells in adult tissue make adult hemoglobin. Presumably, the commitment step occurs at some developmental stage prior to the stem cells which are currently forming colonies in our assay system. We intend to devise techniques suitable for examining stem cells from the earlier developmental stages. Attempts to induce fetal hemoglobin synthesis in young sheep by acute anemic stress and/or erythropoietin injection have to date been unsuccessful. Further in vivo manipulations will include the use of cytotoxic agents to ablate the erythroid stem cell population. We will then examine for the potential for fetal hemoglobin synthesis during regeneration of the erythroid stem cell population.

Human erythroid stem cells also give rise to erythroid colonies in vitro (Individual Project Report: "The Regulation of Erythroid Cell Growth in Culture"). Factors which influence the propensity toward the formation of colonies containing fetal hemoglobin are being sought. To define precisely the role of erythropoietin in inducing cell growth and its relation to the expression of the individual globin genes, we are attempting to purify antibodies to this glyco-protein. Because erythropoietin is not available as a pure immunogen, alternate means to obtain an antibody are being devised. These include the hybridoma technology whereby spleen cells from animals immunized with a crude erythropoietin preparation are fused to myeloma cells in vitro. The resulting hybridoma cells are then assayed for antibodies which destroy the functional activity of erythropoietin (Individual Project Report: "Purification of Erythropoietin"). Similar techniques are being utilized in an effort to obtain antibodies directed toward other growth factors important for erythroid stem cell development.

Many patients with severe congenital or acquired anemias require chronic blood transfusion. Iron accumulation is a life-limiting feature of the transfusion-dependent patient. Currently the only therapeutic approach to patients with thalassemia or other severe anemias requiring regular transfusion is iron chelation to avert or reduce the rate of iron accumulation (Individual Project Report: "Iron Chelation in Transfusional Hemosiderosis"). Our studies have focused on identification of various clinical parameters which may be of utility in defining the extent of iron overload. Both echocardiography and radionuclide cineangiography are useful in defining cardiac anatomy in iron overloaded patients and in detecting subclinical impairment in left ventricular function. Our observations will undoubtedly be useful in determining whether long term treatment with subcutaneous desferrioxamine will prevent the onset of cardiac disease. Ascorbic acid is known to enhance iron excretion in response to desferrioxamine but our earlier clinical observations have suggested that it may be toxic to the iron loaded myocardium. Hence, we are conducting a double blind randomized trial of ascorbic acid in patients currently being treated with subcutaneous desferrioxamine.

The hemoglobin molecule is highly adapted for its primary function in the transport of oxygen to tissue. Hemoglobin-oxygen interaction is therefore fundamental and its manipulation might be of therapeutic importance in various anemias, particularly in sickle cell disease. In this latter disorder, hemoglobin molecules in the deoxy configuration are susceptible to intracellular gelation with resulting deformity and ridigity of individual red cells. This leads to hemolytic anemia and vaso-occlusive crises. By increasing the affinity of SS hemoglobin for oxygen it may be possible to reduce the fraction of molecules in the deoxy configuration and therefore lower the propensity for gelation.

A shift toward lowered oxygen affinity in sickle cell anemia blood is mainly due to intracellular gelation of the abnormal sickle hemoglobin molecules. This gelation is responsible for diminished flow properties of sickle cells and while the lowered oxygen affinity should theoretically improve release of oxygen to the tissues, the diminished flow properties of the cells appears to have the net effect of reducing oxygen delivery. Thus, one would predict that any therapeutically useful strategy would increase the oxygen affinity of sickle cell anemia blood by inhibiting gelation while simultaneously improving oxygen delivery to tissues because of the improved flow properties of the blood.

Measurement of the anaerobic threshold in patients by detection of the rise in lactate concentration in the blood serves as a sensitive measure of oxygen delivery during exercise (Individual Project Report: "Affect of Partial Exchange Transfusion on Oxygen Transport in Sickle Cell Anemia"). To measure the anaerobic threshold non-invasively, we, in collaboration with the Division of Research Services, have devised equipment which gives a breath-by-breath analysis of oxygen consumption and CO_2 production. The increase in CO_2 release which occurs at the anaerobic threshold can be used to define this parameter. We hope to adapt this technology into a clinically useful means to assess clinical severity and judge therapeutic strategies in patients with sickle cell anemia.

The several projects within this Branch are related to the biosynthesis of hemoglobin, the regulation of production of specific hemoglobins in various developmental and experimental states, and the function of hemoglobin in red cells. With this comprehensive approach we hope to provide an experimental basis for an optimal therapeutic approach to various red cell disorders.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02203-07 CHB

PERIOD COVERED October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Molecular Defect in Beta Thalassemia

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

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LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	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)

These studies are directed toward determining the molecular lesion in patients with homozygous β thalassemia. Current evidence indicates that quantitative deficiency of β globin mRNA might result because of defective transcription, ineffective processing, or reduced stability of the final mRNA product. Several avenues of investigation are being pursued. Restriction endonuclease digestion of isolated DNA followed by gel electrophoresis and identification of those fragments containing globin gene sequences has shown that the general structure and organization of the β globin genes is normal in the 25 patients studied. The DNA from two individual patients has been partially digested with Eco RI, linked to the bacteriophage vector, Charon 4A, and cloned into E. coli. Recombinants containing parts of the β globin gene have been identified and are under analysis. Impaired stability of β globin mRNA in one patient seems likely based on equivalent rates of transcription of the α and β globin genes but declining proportions of β globin mRNA during incubation of erythroid cells in vitro. Finally a technique to study processing has been developed whereby the precursor to mature globin mRNA can be quantitated and compared to the concentration of cytoplasmic β globin mRNA.

1101

Objectives: The objective of these studies is to use the naturally occurring model of defective gene expression provided by β thalassemia to attempt to understand gene regulation. Patients with this disorder produce a reduced quantity of a normal polypeptide. 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 provide an approach to specific therapy.

Methods:

1. Restriction endonuclease mapping. High molecular weight DNA is prepared from peripheral blood leukocytes by proteinase K digestion, phenol extraction, dialysis, RNAse digestion, phenol extraction and a final dialysis step. This is digested with specific restriction endonucleases, the resulting DNA fragments are fractionated by agarose gel electrophoresis, the DNA in the gel is transferred to a nitrocellulose filter by "blotting" and the fragments containing globin gene sequences are identified by annealing the filter to a radioactive globin gene probe followed by radioautography.

2. Molecular cloning of human globin genes: High molecular weight human DNA is digested with Eco RI, a fraction corresponding to 10-20 kbs in length is purified by sucrose gradient centrifugation, ligated to the purified arms of Charon 4A, and cloned into E. coli. Recombinants containing globin genes are identified by plaque hybridization assay in which human globin cDNA sequences are used as a probe.

3. Quantitation of radioactive mRNA sequences. Bone marrow cells are fractionated by bouyant density centrifugation to eliminate most of the enucleated red cells. The remaining nucleated cells are incubated in [^{3}H]uridine for periods ranging from 20 minutes to two hours and then chased in cold uridine for periods up to 20 hours. RNA is isolated by extraction in guanidinium hydrochloride followed by bouyant density centrifugation in cesium chloride. Radioactive globin mRNA sequences are quantitated by annealing to cellulose to which has been bound appropriate fragments from recombinant plasmids containing human α, β, or γ globin gene sequences.

4. Quantitation of β globin mRNA precursor: Total cellular RNA is isolated as described above. It is annealed both to α and β cDNA prepared from recombinant plasmids and also to a probe specific for the intervening sequences present in the β globin mRNA precursor. This latter probe is prepared from a recombinant plasmid which contains a Bam HI - Eco RI fragment subcloned into the plasmid from DNA derived from HBG1, a recombinant bacteriophage, containing the human delta and beta globin genes.

Major Findings:

1. Twenty-five β globin genes affected by a thalassemic mutation have been examined by restriction endonuclease analysis. The distribution of intragenic and surrounding Bam HI, Eco RI, and Pst I sites has been found to be

to be normal in all instances. Furthermore, the length of the large intervening sequence in all the globin genes appears to be identical to that found in normals. Thus there are no major structural alterations in the β globin gene in most patients with β^+ thalassemia. In contrast, one homozygote for hereditary persistence of fetal hemoglobin exhibits complete absence of the delta and beta globin genes.

2. In collaboration with the Laboratory of Molecular Hematology, DNA from two thalassemic individuals has been cloned. Screening of 450,000 plaques from one of the resulting genomic libraries has led to the identification of two recombinants containing the embryonic epsilon globin gene and a third which contains the 3' end of the β globin gene. Screening of the second library has led to the identification of a recombinant containing the delta globin gene.

3. Considerable technical difficulty has been encountered in measuring the stability and half life of globin mRNA in thalassemic erythroid cells. This arises because of the self-annealing property of isolated RNA and also an unexpected, non-specific binding of RNA to cellulose during the hybridization reaction. This problem is being circumvented by quantitating radioactive globin mRNA sequences by virtue of their protection from RNAse digestion with purified globin cDNA. One patient has been studied in detail. Alpha and beta globin mRNA transcription appears to be equivalent but prolonged incubation leads to a progressive inbalance in the radioactivity found in β compared to α globin mRNA. These data are most consistent with the primary lesion being instability of β globin mRNA in this particular patient.

4. The assay necessary for quantitation of β globin mRNA precursor has been developed. Because of the high adenosine-thymidine content of the human beta globin gene intervening sequence, it is necessary to perform the annealing reaction at a significantly lower temperature than that usually employed for RNA-DNA hybridization but still sufficiently high to prevent the reannealing of the DNA double stranded probe. The one patient studied to date appears to have a normal concentration of precursor compared to mature cytoplasmic β globin mRNA.

Significance to Biomedical Research in the Institute Program: Homozygous β-thalassemia is a disease which causes serious morbidity and mortality to its victims. Thus an understanding of the genetic basis of this disorder may result in therapy which could be of extraordinary benefit to these individuals. Furthermore, this disease is a prototype of a regulatory human disorder and may provide insight into the mechanism of gene regulation in human cells.

Proposed Course of the Project: Over the course of the next several months we hope to identify and purify a number of beta globin genes by the recombinant DNA technology. Their subsequent analysis will include study of their function by transfer into mouse fibroblasts, mouse erythroleukemia cells, and possibly using the vector, SV-40, into green monkey cells. Structural analysis will include detailed restriction endonuclease mapping and DNA sequencing

when indicated. If the initial data indicating that the primary lesion at least in some patients is in globin mRNA stability, an effort will be made to purify sufficient mRNA from a single patient to permit a determination of its sequence.

Publications:

1. Edward J. Benz, Jr., Johnathan Glass, Jon Pritchard, Diane Hillman, Resy Cavallesco, Elaine Coupal, Bernard G. Forget, Patricia A. Turner, Judith A. Kantor, and Arthur W. Nienhuis. Heterogeneity of Messenger RNA Defects in the Thalassemia Syndromes, Annals N.Y. Acad. of Sci., in press.

2. Edward J. Benz, Jr., Molecular Pathology of The Thalassemia Syndromes. In Nienhuis, A.W. (moderator) Thalassemia Major: Molecular and Clinical Aspects. Ann. Int. Med., in press. 1980

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 H1 02204-07 CHB

PERIOD COVERED
October 1, 1978 to September 30, 1979

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:	J. Barker	Research Geneticist (Cell Biology)	CHB	NHLBI
OTHER:	J.E. Pierce	Chief, Laboratory of Animal Surgery		
	L. Stuart	Chief, Ungulate Section, NIH Animal Center		NHLBI
	D. Buckhold	Veterinary Officer		NHLBI
	M.L. Swenberg	Chemist	CHB	NHLBI
	A.W. Nienhuis	Chief, Clinical Hematology Branch	CHB	NHLBI

COOPERATING UNITS (if any)

Section on Laboratory Medicine and Surgery, NHLBI;
Ungulate Section, NIH Animal Center; Patient Services Department

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The 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 synthesized by sheep erythroid cells. We have found that β^C globin synthesis may be induced by erythropoietin (epo) in erythroid stem cells from all gestational ages beyond 50 days at the expense of either fetal, or late in gestation, β^A globin synthesis. Those colonies derived from primative stem cells make proportionally more β^C globin than do colonies derived from late stem cells. In contrast, in animals lacking the β^C globin gene, epo concentration appears to have no role in modulating the proportion of γ and β globin synthesis in erythroid colonies in vitro. Furthermore, acute anemia and epo injection in young lambs fails to induce γ globin synthesis. The ratios of β^C and β^A globin synthesis during erythroid maturation appear to be stable in vivo but we found that the fraction of β^C globin synthesis in mature colonies was greater than that found in primative colonies. Thymectomy at 60-80 days resulted in the expected lymphopenia at birth but had no effect on the pattern of hemoglobin switching during the perinatal period.

1105

Objectives: The objective of this project is to determine the normal mechanism of regulation of expression of globin genes during ontogeny in sheep. Analysis of erythroid colony formation from erythroid stem cells at various stages of differentiation and the influence of various exogenous factors on the pattern of hemoglobin synthesis by these colonies is the general experimental approach. Also, surgical manipulation or injection of various substances in animals at various developmental stages form the basis for an attempt to understand and manipulate the pattern of hemoglobin switching in vivo.

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 γ globin gene (Hb F = $\alpha_2\gamma_2$) is expressed during fetal life while the allelic adult β globin genes (β^A and β^B) are expressed in adult animals making the normal adult hemoglobins, Hb A ($\alpha_2\beta^A_2$) and Hb B ($\alpha_2\beta^B_2$). The β^C globin gene (Hb C = $\alpha_2\beta^C_2$) is expressed transiently in newborn animals and in adults made anemic. Induction of β^C globin synthesis is specifically stimulated by erythropoietin (epo) both in vivo and in vitro.

The epo induced synthesis of Hb C in both fetal and adult sheep erythroid precursors has been characterized at the cellular level. This switch has been compared with the switch from fetal to adult hemoglobin synthesis which occurs during late gestation. Attempts have also been made to reinitiate fetal hemoglobin synthesis with epo in lambs lacking a gene for β^C globin. Since cellular interactions between T cells and erythroid precursors appear to play a role in the generation of red blood cells, the role of thymocytes in switching mechanisms has been examined. The effect of time after epo stimulated commitment of Hb C synthesis on the ratio of Hb C/Hb A in erythroblasts at various stages has been analyzed.

Methods:

Erythroid precursors are collected from bone marrow of young lambs or from fetal liver or bone marrow. The donors are either untreated, thymectomized or bled to hematocrits of 15 to 20 percent and/or injected with epo. The cells are routinely fractionated by centrifugation in Ficoll-metrizamide solution. Further segregation of different classes of erythroid progenitors is accomplished by unit gravity sedimentation in bovine serum albumin gradients. Cells are grown in methylcellulose or plasma clot cultures in varying concentration of epo. Globin synthesis is monitored by adding [^{3}H]leucine to cultures 24 hrs before incubation at 37^o is terminated followed by chromotographic resolutions of the globins on ion-exchange column.

Major Findings:

1. Comparison of epo induced Hb C synthesis in fetal erythroid colonies to the developmental switch from Hb F to Hb A synthesis. Cells from fetuses of 60-124 day's gestation either with (Hb A homozygotes) or without (Hb B homozygotes) the gene for Hb C were grown in vitro at high or low epo concentrations. Hb C synthesis was induced in all cultures from fetuses homozygous for Hb A maintained at high but not at low epo levels. In cells lacking

the Hb C gene, the switch from fetal to adult hemoglobin was not stimulated by epo. Adult hemoglobin amounted to less than 10% of the total in cultures of cells from fetuses of 51 and 89 day's gestation, whereas, the Hb C switch was always greater than 50% at all ages studied. A switch from predominantly Hb F to predominantly Hb B was observed in vitro in colonies derived from stem cells obtained from a 124 day animal. This switch correlated temporally with the expected development switch and was not influenced by epo concentration.

2. Attempts to reinitiate Hb F synthesis in young lambs: Lambs homozygous for Hb B were maintained at a low hematocrit and injected with epo. At various intervals after induction of anemia, bone marrow cells were aspirated and grown in vitro. No Hb F synthesis or mRNA for γ globin was found after up to 7 days in vivo and an additional 5 days in vitro. Thus increased epo concentration is not sufficient to induce an adult to fetal hemoglobin switch. Exposure of human cells to epo in vitro results in augmented fetal Hb synthesis. Unlike sheep, human cells produce 1-2% fetal Hb after birth. The difference between sheep and human may depend on the capacity of cells producing some human fetal Hb to be selectively expanded in vitro.

3. Cellular interactions and switching mechanisms. Thymectomy was performed on fetal sheep between 60 and 90 day's gestation. The proportion of fetal and adult hemoglobin synthesis was monitored by cellulose acetate electrophoresis of hemolysates obtained from birth to two months of age. White cell counts were performed and the hematocrits and animal weight recorded. There appeared to be no significant difference between thymectomized animals and their normal litter mates as to amount of Hb A at birth, the disappearance of Hb F after birth or the onset of Hb C synthesis. Lymphopenia at birth was noted in the thymectomized animals. These data suggest that the thymus plays no role in hemoglobin switching after mid-gestation.

4. The ratio of Hb A and Hb C synthesis during erythroid maturation: A lamb was injected with epo and bone marrow aspirates were removed at 36, 48, and 60 hours thereafter. The cells were fractionated by sedimentation on bovine serum albumin gradients. Populations of pro-and basophilic erythroblasts, polychromatophilic erythroblasts and reticulocytes were exposed to (^{3}H)leucine in vitro for 6 hours. The globins synthesized in each fraction were analyzed by ion exchange chromatography. The data obtained in this experiment indicated that the proportion of synthesis of the two hemoglobins did not change during erythroid maturation but reflected commitment at the stem cell stage with regard to the relative expression of the β^A and β^C genes.

5. Hemoglobin synthetic pattern during maturation of erythroid colonies: Cells from fetal liver were grown in methylcellulose at high concentrations of epo. At 4 to 6 days in vitro the less differentiated (pale) colonies were separated from the well-differentiated (red) colonies. Individual colonies were plucked from the methylcellulose with a capillary pipet, pooled as to microscopic classification, and exposed to [^{3}H]leucine. The proportion of γ and β^C globin synthesis by the immature and mature colonies was compared to globins made by the unsegregated colonies. The data from this preliminary ex-

periment suggested that the proportion of β^C globin synthesis increases during colony maturation.

6. Antibodies specific for Hb F, Hb C, and Hb A have been prepared using immunoabsorption techniques. These will be used in radioimmunoassays to quantitate the pattern of hemoglobin switching in vivo and in vitro.

Significance to Biomedical Research and the Program in the Institute: Several human anemias (e.g. beta thalassemia, and sickle cell anemia) are characterized by normal α globin synthesis and abnormal β globin synthesis or function. A common feature of these anemias is that synthesis of fetal hemoglobins is normal 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 β globin synthesis is therefore the therapeutic reutilization 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 data appear to suggest that the stem cells from either fetuses or adults are commited with regard to their pattern of hemoglobin synthesis. Therefore, to look into the mechanism of regulation of fetal and adult hemoglobin synthesis, it will be necessary to develop culture techniques suitable for propagation of stem cells from earlier developmental stages. Growth factors derived from mononuclear cells, presumably T-lymphocytes, are necessary in other species for the development of these early stem cells in vitro. Such interactions will be sought in sheep erythropoieses. The pattern of hemoglobin synthesis during colony maturation will be further explored by devising techniques suitable for measurement of hemoglobins produced in individual colonies. Further attempts to induce Hb F synthesis in young lambs will utilize cytotoxic agents to destroy the developing erythron. During regeneration, evidence of Hb F synthesis in vivo and the development of stem cells capable of making Hb F in vitro will be sought.

Publications

1. Nienhuis, A.W., Barker, J.E., Benz, E.J., Jr., Croissant, R.D., Kantor, J. Kretschmer, P., Miller, D.M., and Young, N.S. Regulation of hemoglobin switching in sheep: Cellular and molecular aspects. ICN-UNCLA Symposium, pp. 91-108, 1978.

2. Barker, J.E., Pierce, J.E. and Nienhuis, A.W.: Stimulation of hemoglobin C synthesis by erythropoietin in fetal and neonatal sheep. In Stamatoyannopoulos, G., Nienhuis, A.W. (Eds.) Cellular and Molecular Regulation of Hemoglobin Switching. New York, Grune and Stratton, Inc., 1979, pp. 179-192.

3. Nienhuis, A.W., Croissant R., and Barker, J.E.: Induction of Hemoglobin C synthesis in sheep: Characterization of the "Switching" Stem Cell. In Stamatoyannopoulos, G., Nienhuis, A.W. (Eds.) Cellular and Molecular Regulation of Hemoglobin Switching. New York, Grune and Stratton, Inc. 1979, pp 397-420.

4. Nienhuis, A.W. and Stamatoyannopoulos, G., Hemoglobin Switching: Meeting Review, CELL 15:307-315, 1978.

5. Nienhuis, A.W., Barker, J.E., and Croissant, R.D. Overview: Mechanism of Regulation of Hemoglobin Synthesis at the Cellular Level. N.Y. Acad. of Sci., N.Y. In press

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02206-06 CHB

PERIOD COVERED

October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Regulation of the Sheep Globin Genes

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

PI:	P. Kretschmer	Visiting Expert	LMH, NHLBI
OTHERS:	R. Kaufman	Research Hematologist	CHB, NHLBI
	H. Coon	Biologist	LMH, NHLBI
	J. Chen	Senior Staff Fellow	CHB, NHLBI
	C.E. Geist	Chemist	CHB, NHLBI
	P.H. Turner	Medical Technologist	CHB, NHLBI
	A.W. Nienhuis	Chief	CHB, NHLBI

COOPERATING UNITS (if any)

Molecular Hematology Branch, NHLBI

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1.5	0.5

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The purpose of this project is to define the mechanism of regulation of the individual globin genes during erythroid differentiation. Total genomic DNA from sheep, homozygous for the B^A globin gene, has been cut into fragments of 10-20 kb in length by restriction endonuclease digestion and cloned into E. coli using the vector, bacteriophage Charon 4A. Individual recombinants containing globin gene sequences have been identified. The B^A, γ, and epsilon globin genes have been characterized; each contains a large and small sequence. The presence of a restriction endonuclease site for Pvu II at the 5' end of the gene suggests sequence homology in this region. Further analysis of these isolated globin genes is planned in an attempt to determine the role of DNA sequence in regulating hemoglobin switching.

1110

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. Cloning of sheep genomic DNA sequences containing globin genes and surrounding sequences: High molecular weight DNA is purified from fetal liver cells by proteolytic digestion, phenol extraction, and RNAse digestion. Following partial Eco RI digestion, 14 to 20 kilobase (KB) fragments of this genomic DNA are ligated to DNA of the bacteriophage cloning vector Charon 4A. Recombinant DNA molecules are mixed with previously prepared bacterial extracts (which contain all the proteins necessary to form mature bacteriophage particles), and packaged *in vitro*. The resulting bacteriophage "library" of 15-20 KB genomic sequences can then be screened, using radioactively labelled probes, for globin gene sequences. Clones that yield positive signals on the primary screen are picked and purified as single plaques in a secondary screen. In order to identify the globin gene sequences contained within these clones, bacteriophage DNA is extracted, digested with restriction endonucleases, and subjected to agarose gel electrophoresis. The resulting restriction fragments are transferred to nitrocellulose filters and those fragments containing globin gene sequences are identified following hybridization of this filter to a radioactive probe. In this manner, a restriction endonuclease map of the clone can be deduced.

2. Detailed analysis of globin gene-containing bacteriophage clones;

a. Restriction endonuclease analysis: The restriction endonuclease map of the clone, deduced as above, is compared with that of a synthetic cDNA

sheep globin gene. Differences and similarities between the two maps can indicate the identity of globin gene within the clone.

b. Hybridization analysis: DNA, isolated from the clone, is hybridized to radioactive cDNA made from RNA preparations containing known globin gene sequences. The melting temperatures of duplexes which form can be used to identify the globin gene contained within each clone.

c. DNA sequence analysis: After a restriction endonuclease map of an individual recombinant is obtained, specific restriction endonuclease fragments are isolated. Individual fragments are labelled on the 5' ends by the polynucleotide kinase reaction, cut again with a restriction endonuclease, and the two 5' ends separated by polyacrylamide, gel electrophoresis. The DNA nucleotide sequence can be obtained by partial chemical degradation of the individual 5' 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.

MAJOR FINDINGS:

1. A genomic library of a million unique bacteriophage clones containing DNA from a sheep homozygous for the β^A gene has been derived. Eight hundred and fifty thousand of these clones were screened for sequences homologous to either β^C or γ cDNA and nine containing globin gene sequence have been identified. Of these, three have been characterized in detail using methods described above. The clone SG-21 contains a complete β^A sheep globin gene with the total insert size being 19 kb. This β^A gene has been shown to contain a large (900 bp) and small (105 bp) intervening sequence at intragenic positions similar to those of other adult genes so-far studied in humans, mice and rabbits. Clone SG-31 contains a full-length sheep γ gene positioned in the middle of the total insert length of 12 kb, and has been demonstrated to have a large intervening sequence of similar size and position to that in the β^A gene. Clone SG-4 has been demonstrated by hybridization experiments with embryonic cDNA, to be a complete β-like embryonic sheep globin gene contained within 12 kb of insert. Similar to the adult β^A and γ genes, it has a large intervening sequence demonstrated to be at least 750 kb in size.

2. A detailed restriction endonuclease map of the 5' end of the beta^A globin gene, including the site at which transcription is thought to begin and the small intervening sequence, has been deduced. Individual fragments have been isolated, labelled on the 5' end, and are being used for the sequence analysis.

3. In collaboration with members of the Pulmonary Branch, the sheep library constructed as described above, was screened for clones containing sequences homologous to collagen cDNA. Of 150,000 recombinants screened, four were found to contain collagen gene sequences. Characterization of these clones is described in detail in the Pulmonary Branch report #4.

PROPOSED COURSE OF PROJECT:

Further sheep genomic globin genes, especially those corresponding to the β^C gene, will be isolated. In order to completely define the 40-60 kb genomic region anticipated to code for all beta-like sheep globin genes (embryonic, fetal and adult), the sheep library will be screened for sequences homologous to the ends of the globin gene clones already isolated, thus "walking" along the chromosome in this region. Characterization and DNA sequence analysis of homologous regions surrounding the individual globin genes will be made in an attempt to more fully understand regulation of globin gene switching in sheep.

The genes and their flanking regions themselves will be utilized to prepare affinity columns suitable for purifying specific nuclear proteins which may serve as transcriptional regulatory factors. An effort will be made to devise a reconstituted system using the cloned globin genes from genomic DNA, histones, and specific nuclear protein molecules to produce a transcriptionally active chromatin complex.

PUBLICATIONS:

1. Benz, E.J., Jr., Barker, J.E., Pierce, J.E., Turner, P.A. and Nienhuis, A. W.: Hemoglobin switching in sheep: Commitment of erythroid stem cells to expression of the β^C-globin gene and accumulation of β^C-globin mRNA. Cell 14:733-740, 1978.

2. Benz, E.J., Jr., Steggles, A.W., Geist, C. and Nienhuis, A.W.: Hemoglobin switching in sheep: Quantitation of the β^A and β^C mRNA sequences in nuclear and cytoplasmic RNA during the Hb A to Hb C switch. J. Biol. Chem. 253:5025-5032, 1978.

3. Nienhuis, A.W., Axelrod, D., Barker, J.E., Benz, E.J., Jr., Croissant, R., Miller, D., and Young, N.: Regulation of the individual globin genes, reprinted from Differentiation of Normal and Neoplastic Hematopoietic Cells. In B. Clarkson, P.A. Marks and J.E. Till (Eds.): Cold Spring Harbor Conference on Cell Proliferation, New York, Cold Spring Harbor Laboratories, 1978, Vol. 5, pp. 295-310.

4. Benz, E.J., Jr., Kretschmer, P.J., Geist, C.E., Kantor, J.A., Turner, P.A. and Nienhuis, A.W.: Hemoglobin switching in sheep: Synthesis, cloning, and characterization of DNA sequences coding for the β^B, β^C and γ globin mRNAs. J. Biol. Chem., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-02207-06 CHB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Globin Gene Expression in Somatic Cell Hybrids

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

PI:

A.W. Nienhuis	Branch Chief	CHB	NHLBI
W.F. Anderson	Laboratory Chief	LMH	NHLBI

Other:

M. Willing	Graduate Student	LMH	NHLBI
P. Turner	Medical Technologist	CHB	NHLBI
N. Young	Visiting Expert	CHB	NHLBI

COOPERATING UNITS (if any)

Laboratory of Molecular Hematology, NHLBI

LAB/BRANCH
Clinical Hematology Branch

SECTION

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NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Several somatic cell hybrids formed by fusion of mouse erythroleukemia cells and human fibroblasts and known to contain human chromosome 11 by virtue of the presence of human LDH-A, were analyzed for their globin gene and mRNA content. The human gamma and beta genes were identified but only beta and not gamma mRNA was found in the cytoplasm. A low level of human beta globin production was identified in these hybrid cells by a sensitive radioimmunoassay.

1114

Objectives:

The goal of this work during the past 12 months has been to characterize hybrids between mouse erythroleukemia cells and various human cell populations, which contain the human β and γ genes. Expression of the β but not γ genes has been further analyzed. An attempt to determine the structure of the γ and β genes in chromatin using DNAse I as a probe has been conducted. A sensitive radioimmunoassay for human β globin has been applied to determine whether this product is present in these hybrid cells.

Methods:

1. The formation and characterization of the somatic cell hybrids has been performed in the Molecular Hematology Laboratory and is described in their project report (Z01-HL-02202-02 MH).

2. Analysis of globin gene in mRNA content: Total cytoplasmic RNA or nuclear DNA is prepared and annealed to pure α, β, orγ cDNA probe derived from recombinant plasmids.

3. Radioimmunoassay for β globin: An antisera specific for the human hemoglobins A and F has been raised in goats. In a sensitive radioimmunodisplacement assay, this antisera has been shown to be capable of detecting very low levels of the human β globin but cross reacts minimally with the mouse hemoglobin molecules.

Major Findings:

1. Consistent expression of the human β but not γ globin gene in hybrid cells containing chromosome 11 has been defined.

2. Low levels of human β globin production has been found in these cells although it is proportionately small compared to the concentration of β globin mRNA in the cytoplasm.

Significance to Biomedical Research and Institute Program:

An understanding of the mechanism of mammalian gene regulation is of fundamental importance in understanding human genetic disease. This program offers an opportunity for insight into genetic regulation by exploring our knowledge of the expression of the globin genes in somatic cell hybrids.

Proposed Course of This Project:

Because this avenue of experimentation is being explored primarily by workers in the Laboratory of Molecular Hematology with occassional collaboration by the Clinical Hematology Branch, further details of this study will be described in the Annual Reports of the former laboratory. Therefore, this project is terminated.

Publications:

1. Willing, M.C., Nienhuis, A.W., and Anderson, W.F.: Selective activation of the human beta but not gamma globin gene in human fibroblast X mouse erythroleukemia cell hybrids, Nature, Vol. 277, No. 5697, pp. 534-538, Feb. 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02208-05 CHB

PERIOD COVERED

October 1, 1978 through September 30, 1979

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,	Chief	CHB	NHLBI
OTHER:	Patricia Griffith,	Clinical Nurse Specialist	CHB	NHLBI
	M. Leon,	Clinical Associate	CB	NHLBI
	G. Strewler,		K&E	NHLBI
	J. Borer,	Senior Investigator	CB	NHLBI
	H. Strawczynski,	Montreal Childrens Hospital, Montreal, Quebec, Canada		
	W.F. Anderson,	Chief	LMH	NHLBI

COOPERATING UNITS (if any)

Cardiology Branch, NHLBI; Laboratory of Molecular Hematology, NHLBI;

Thalassemia Clinic, Montreal Childrens Hospital, Montreal, Quebec, Canada

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

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)

These studies are designed to evaluate the clinical benefits achieved by iron chelation in patients with chronic iron overload. Detailed assessment of cardiac, endocrine and hepatic function are performed prior to initiation of chelation therapy and these analyses are repeated annually during the course of iron removal. Desferrioxamine is administered by subcutaneous infusion and the amount of iron removed is determined by quantitation of urinary iron excretion and careful recording of total iron administered by transfusion. Those patients who have no evidence of cardiac disease are randomized to receive either ascorbic acid (3 mg/kg) or placebo. Cardiac function is assessed by 24 hour recordings of the cardiac rhythm, echocardiography, and radionuclide cineangiography as well as standard clinical techniques. Radionuclide cineangiography during exercise provides a very sensitive index of cardiac function in that 13 of 16 patients who received over 100 units of blood failed to increase their ejection fraction during exercise in the normal fashion.

1117

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: Estimate of cardiac size by chest x-ray and electrocardiographic analysis is obtained. Echocardiographic studies are obtained to determine anatomical dimensions of the left ventricle and left ventricular function as assessed by resting ejection fraction. In addition, the configuration of the left ventricle and resting ejection fraction is determined by radionuclide cineangiography. Supine exercise is performed to 70% of maximal predicted heart rate and the ejection fraction is again determined by radionuclide cineangiography. By these methods a detailed analysis of cardiac structure and function is achieved.

2) Endocrine evaluation includes specific testing of the pituitary, thyroid, adrenal, pancreatic islets, and gonad function by baseline measurements and various provocative tests.

3) Liver function is determined by standard clinical testing. In addition, liver biopsies are performed to assess histology and to quantitate liver iron concentration.

4) Serial serum ferritin measurements are obtained to assess the utility of this parameter in estimating total body iron stores and also to follow the course of iron removal.

MAJOR FINDINGS:

1. One hundred and four patients have received the initial evaluation in determining the severity of iron overload and the extent of functional derrangement in specific target organs. These patients range in age of 6 to 71 years; 61 have homozygous β thalassemia and 43 are adults with various types of acquired transfusion dependent anemia or primary hemochromatosis. Of the

patients in the first category, 41 were randomized to the ascorbic acid trial. Most of the others were excluded because of cardiac disease although two patients refused this phase of the study.

2. The only endocrine abnormality detected frequently is impairment in gonadal function evident as patients reach the stage of normal puberty. This lesion is usually due to failure of production of gonadotropins by the pituitary gland.

3. Twenty-four patients have been evaluated by radionuclide cineangiography. Eight who had received fewer than 100 transfusions had a normal ejection fraction both at rest and during exercise, whereas only three of the 16 patients who had received over 100 units of blood had normal responses during exercise.

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 PROJECT:

The project will be continued until a suitable iron chelator is found and evaluated or until the need for transfusion in thalassemia and other congenital hemolytic anemias is removed. Currently we are accumulating a series of 55-60 patients of various ages who are treated intensively with chronic subcutaneous desferrioxamine. Since the natural history of progressive iron overload in patients with congenital transfusion dependent anemia is well established, we will attempt to determine whether any deviation in the incidence of cardiac, endocrine, and hepatic complications ensues from this therapeutic effort. The randomized trial to evaluate the use of low dose ascorbic acid to enhance iron excretion will be completed. For cardiac evaluation we will focus on the use of radionuclide cineangiography because this clearly provides the most sensitive technique to assess the function of the heart.

PUBLICATIONS:

1. Nienhuis, A.W., Griffith, P., Henry, W., Borer, J., Leon, M. and Anderson, W.F.: Evaluation of cardiac function in patients with thalassemia major. Annals N.Y. Acad. Sci., in press.

2. Nienhuis, A.W., Benz, E.J., Jr., Propper, R., Corash, L., Henry, W., Borer, J. and Anderson, W.F.: Thalassemia major: molecular and clinical aspects. Ann. Int. Med. in press.

3. Young, N., Henry, W., Nienhuis, A.W.: Treatment of primary hemochromatosis with deferoxamine. J. Am. Med. Assoc., 241:1152-1154, 1979.

4. Nienhuis, A.W. and Propper, P.D.: The thalassemias: disorders of hemoglobin synthesis. In Hematology of Infancy and Childhood. (D.G. Nathan and F. Oski, eds) New York, Saunders, in press, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-02210-04 CHB

PERIOD COVERED
October 1, 1978 through August 30, 1979

TITLE OF PROJECT (80 characters or less)

Regulation of the Respiratory Function of Blood

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

PI:

R.M. Winslow	Senior Investigator	CHB NHLBI

Other:

N. Statham	Biologist	CHB NHLBI
M. Samaja	Visiting Scientist	University of Milan Milan, Italy
L. Rossi-Bernardi	Prof. Enzymology, Univ. of Milan, Milan, Italy	
L. Thibault	Engineer	BEIB

COOPERATING UNITS (if any) Division of Research Services, Biomedical Engineering and Instrumentation Branch, and University of Milan

LAB/BRANCH
Clinical Hematology Branch

SECTION

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)

The objective of this project is to quantitate and understand the molecular and cellular regulation of hemoglobin function within red cells. Analysis of blood from normal and abnormal individuals as well as individuals with mutant human hemoglobins are performed using oxygen equilibrium measurements on whole blood and purified hemoglobin solutions.

1121

Methods:

1. The automatic continuous oxygenation apparatus previously described has been further improved and further automated. It is presently operated by a very inexpensive micropressor system which is suitable for use under field conditions. It is completely independent of the NIH computer system although that system can be accessed when needed. The cuvette has been further modified to allow the precise measurement of temperature during the oxygenation.

2. A variation of the reaction cuvette has been developed by BEIB. This method does not use the hydrogen peroxide principle, but rather the exchange of oxygen across a silicone rubber membrane. The advantage of this method is that pure hemoglobin solutions at very high concentrations can be studied and the dissociation curve can be measured in both ascending and descending fashions. This development has particular application in the problem of measurement of oxygen affinity of sickle cell anemia blood and concentrated hemoglobin solutions. At present, the method has been successfully used in normal and sickle cell anemia samples. In its present state of development it is capable of measuring p50, the oxygen pressure at half-saturation, which agree very well with the values we obtained using the hydrogen peroxide system.

3. A third method of measuring the descending oxygen dissociation curve has been developed, using beef heart mitochondria to consume oxygen. In this way, the descending curve of sickle cell blood can be measured.

Findings:

1. The precise dependence of OEC on temperature has been defined.

2. The precise dependence of the OEC on pH is nearly complete. This set of data, the result of nearly 2 years of work, will define the position of the OEC at all saturations (previous work has been concerned only with p50) so that mathametical analysis of blood and respiratory function can now begin.

Significance to Biomedical Research and the Program of the Institute:

The delivery of oxygen to tissues is the primary respiratory function of the blood. The present studies are aimed at assessing the importance and mode of regulation of whole blood oxygen affinity in this overall process. To this end, we are undertaking a quantitation of the effects of the various mediators of whole blood oxygen affinity such as CO_2, pH, 2,3-DPG, and temperature. These studies can further our understanding of adaptive mechanisms which are employed during anemia, respiratory disease, high altitude, and in patients with abnormal hemoglobin which affect the ability of the red cell to deliver oxygen to tissues. Certain of these adaptive mechanisms are common to all situations and some are unique to others.

Proposed Course of the Project: Because Dr. Winslow is leaving the NIH in September this project will be terminated.

Publications:

1. Samaja, M., Winslow, R.M.: The separate effect of H^+ and 2,3-DPG on the oxygen equilibrium curve of human blood. British Journal of Hematology. 41: 373-381, March, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1-HL-02300-03 CHB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Structure of the Globin Genes in Chromatin

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

PI:	Richard Croissant	Staff Fellow	CHB	NHLBI
	Neal S. Young	Senior Investigator	CHB	NHLBI
Other:	Arthur W. Nienhuis	Branch Chief	CHB	NHLBI
	Peter Kretschmer	Visiting Fellow	LMH	NHLBI

COOPERATING UNITS (if any)
Molecular Hematology Laboratory

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	1.25	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The goal of this work is to determine the structural organization of the individual globin genes in chromatin. Pancreatic DNAse I is used as a probe of chromatin structure since transcriptionally active genes in isolated nuclei have been shown to be exquisitely sensitive to this enzyme. Prior studies have shown that the γ globin gene is in the open conformation in fetal erythroid cells from sheep but the adult β globin genes are insensitive to DNAse I. The adult β globin genes are activated during the developmental switch but the γ gene also remains in the active conformation. Maturation of mouse erythroleukemia cells in vitro is also not associated with a major change in globin gene conformation. These studies are being extended to include the DNA sequences around and between the individual globin genes. Cloned DNA segments including mouse, human, and sheep globin genes are sources of probes both for the coding sequences and also the flanking sequences of these genes.

1124

Objectives:

Only a restricted portion of the total DNA sequences in individual cells are expressed. Primary regulation of these sequences is imposed by their individual structure in chromatin. Chromatin is composed of histones arranged in "nu bodies" which take the forms of beads strung on the DNA double helix. Non-histone proteins are also found in chromatin and may be concentrated in the transcriptionally active fraction although they are also associated with "nu bodies". The structural arrangement of DNA sequences in chromatin results in only 6-10% of individual genes being available for transcription in specific differentiated cells. The goal of this project is to determine the structural differences of the individual globin genes in chromatin. Furthermore, we hope to learn the structural basis for inclusion of particular genes into the actively transcribed fraction of nuclear chromatin. Identification of specific proteins may lead us to the putative transcriptional regulatory factors which may determine the structure of the gene in chromatin or modulate its transcriptional rate.

Preliminary experiments have suggested an equal susceptibility of the β and γ globin genes to DNAse I digestion in nuclei from human erythroid bone marrow. Further experiments will address two questions: is the structure of the globin genes in human fetal liver different from that in adult bone marrow and are the globin genes in the same conformation in erythroid bone marrow from patients with different diseases, including the β thalassemias?

Our results to date have been obtained using cDNA probes that hybridize only to that portion of the globin gene which codes for functional processed mRNA transcript. No information is provided about the conformational state of flanking sequences particularly during regulatory events. RNA polymerase initiates transcription of the β globin gene region approximately 100 bp distal to the sequences detected with cDNA. Experiments designed to examine the DNAse I sensitivity of flanking regions containing initiation, intervening, and termination sequences are the next step. Hybridization probes for these sequences are available from recombinant DNA plasmids containing mouse, human, or sheep globin genes. The principal experimental strategy is to determine the survival of specific restriction endonuclease fragments (or restriction sites) in DNA isolated from intact nuclei previously exposed to brief DNAse I digestion.

Methods:

1. Nuclei have been prepared from erythroid tissues, including cells in culture, sheep fetal liver and bone marrow, and human bone marrow as well as non-erythroid tissues, including sheep liver and spleen and human myeloid bone marrow. In some instances, separation of erythroid cells into early and late fractions by Ficoll-Hypaque isopycnic density gradient centrifugation, followed by unit gravity sedimentation in albumin solution, has been performed.

2. Nuclei have been exposed to DNAse I in order to release 1-20% of the

total nucleic acids. DNA remaining following digestion, as well as DNA from control nuclei, is purified by removal of proteins and RNA. The DNA obtained from the experiments has been hybridized to both synthetic cDNAs, prepared from sheep, mouse, and human mRNA by the reverse transcriptase reaction, and, in some instances, to plasmid-derived cDNA prepared from recombinant plasmids containing one of the sheep or human globin genes. DNA excess and cDNA excess hybridization analysis have been performed, both of which provide a sensitive measure of the quantity of globin gene sequences remaining after exposure of nuclei to DNAse I.

3. To examine the DNAse I sensitivity of sequences flanking the globin genes, isolated DNA from control and DNAse I digested nuclei are incubated with various restriction endonucleases. The DNA fragments are displayed on the basis of size by agarose gel electropheresis. Transfer of the DNA to nitrocellulose filters and hybridization to specific radioactive DNA fragments derived from recombinant plasmids will be used to determine the extent of DNAse I sensitivity. Alternatively, probe fragments will be labelled on the 5' terminii, the two strands separated by gel electropheresis, annealed to control and DNAse I digested DNA, and the reconstitution of specific restriction endonuclease sites examined.

Results:

1. DNAse I digestion of nuclei from a large number of bone marrow samples obtained from patients with a variety of hematologic disorders characterized by erythroid hyperplasia, including the beta thalassemias, sideroblastic anemia, and sickle cell anemia has been performed. Solution hybridizations in DNA excess and in cDNA excess have shown that DNAse I digestion of nuclei reduces the concentration of both β and γ gene sequences by 20-50% in erythroid bone marrow tissue. While digestion of the two genes is equivalent, and these results are compatible with those obtained for the sheep globin genes, the degree of digestion of the human globin genes is not as great as in sheep nuclei. There is no correlation between sensitivity to DNAse I of the γ gene and hemoglobin F production in vivo. As in the sheep, these results suggest that selective expression of the β but not the γ gene in the adult organism is not dependent on major structural differences in their conformation in chromatin.

2. Experiments designed to compare the chromatin structure of C-19 mouse erythroleukemic cells before and after DMSO induction of β-major globin mRNA synthesis are in progress. A 7 kb genomic fragment containing the β-major gene was isolated from a previously characterized λ recombinant (MβG2-λWes provided by Dr. P. Leder). This fragment was cut with Hind III restriction endonuclease into three fragments suitable for hybridization studies of the 3' and 5' flanking sequences adjacent to the β major gene in genomic DNA. Each of these fragments was recloned into the plasmid pBR-322. Initial growth of these plasmids in χ-1776 produced insufficient yields and each plasmid was transferred to wild-type E. Coli (C-600). Good yields were obtained and approximately 200 μg of each Hind III fragment was isolated. Two of the fragments containing the immediate 3' and 5' flanking sequences were proved authentic by restriction analysis. Uninduced C-19 cells were

grown in mass culture, nuclei isolated and rates of DNAse I digestion measured. Milligram quantities of control DNA and DNA digested from 2 to 30% were prepared. These DNAs were restriction cut with Hpa I and Hind III alone and sequentially. Electrophoresis and blotting of these samples was successful, but problems with probe specific activity and specificity prevents immediate interpretation. New preparations of labelled probes are being made and DNAse I digestion of nuclei from cells grown in DMSO is in progress. Methods to examine the survival of specific restriction sites following DNAse I digestion are being developed.

3. Experiments to compare the DNAse I fine structure of early and late erythropoietic precursor cells from embryonic mouse liver are in progress. These experiments are conceptually identically to those described for C-19 erythroleukemic cells. Suspensions of liver cells were separated by equilibrium centrifugation into early and late precursors. Late cell preparations synthesize approximately 4 times as much hemoglobin as early cells (per cell). Experiments are in progress to determine, with plasmid probes, whether or not mRNA levels are also different. If so, DNA structural differences between the two populations will be studied by DNAse I sensitivity.

Significance to Biomedical Research and Program in the Institute:

The mechanism by which eukaryotic cells come to express specific genes has not yet been defined. Specifically the role of chromatin structure in modulating gene expression demands further exploration. By these studies we hope to obtain further insight into such human diseases as β thalassemia and sickle cell disease and perhaps gain an appreciation of means which can potentially be employed to induce fetal hemoglobin synthesis in patients with these disorders.

Proposed Course of the Project:

Our immediate goal is to define the size of the transcriptional units surrounding each of the individual globin genes in mouse, sheep and human cells. The availability of cloned probes for embryonic, γ and the β globin genes from these species make this a feasible undertaking. Once this data is available, we will redirect our efforts toward isolating those proteins which are important in establishing this chromatin structural pattern.

Publications:

1. Miller, D.M.. P. Turner, A.W. Nienhuis, D.E. Axelrad and T.V. Gopalakrishnan. Active conformation of the globin genes in uninduced and induced mouse erythroleukemia cells. Cell, Vol. 14:511-521, July, 1978.

2. Young, N.S., Benz, E.J., Jr., Kantor, J.A., Kretschmer, P. and Nienhuis, A.W., Hemoglobin switching in sheep: the γ gene is in the active conformation in fetal liver. The β and γ gene is in the active conformation in fetal liver. The β and γ genes are in the active conformation in bone marrow. Proc. Nat'l. Acad. Sci. USA 75:5884-5888, 1978.

3. Nienhuis, A.W., Benz, E.J., Jr., Miller, D.M., Steggles, A. and Young, N.S.: Chromatin structure and transcription of the globin genes, in "Current Concepts of the Structure and Function of DNA Chromatin, and Chromosomes, Eds. A.S. Dion, Miami, Symposia Specialists, 1979, pp 211-228.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-02301-03

PERIOD COVERED

October 1, 1978 through September 20, 1979

TITLE OF PROJECT (80 characters or less)

Effect of Partial Exchange Transfusion in Oxygen Transport in Sickle Cell Anemia

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

PI:

R.M. Winslow	Senior Investigator	CHB	NHLBI

Other:

N.J. Statham	Biologist	CHB	NHBHI
H. Klein	Assistant Chief	CC	BB
F. Brown	Clinical Associate	CHB	NHLBI
A. Nienhuis	Branch Chief	CHB	NHLBI

COOPERATING UNITS (if any)

Blood Bank, Clinical Center, NIH

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1-1/2	1/2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Partial exchange transfusion is performed frequently in patients with sickle cell anemia when surgery is indicated, when life-threatening complications (for example bilobar pneumonia) occur, or when a patient is totally incapacitated by recurring crises. Because either fresh or stored blood has higher oxygen affinity then sickle cell blood, infusion of large amounts of such blood could cause dramatic alteration of the recipient's blood oxygen affinity. The present study has indicated that partial exchange transfusion with maintenance of hemoglobin concentration and blood volume results in improved exercise tolerance. We interpret this to mean that the flow properties of sickle cell blood through peripheral capillary beds may be more important in determining oxygen delivery than oxygen affinity of the red cells themselves.

1129

Methods:

1. Partial exchange transfusion is achieved in adult patients with sickle cell anemia using the Hemonetics blood cell separator in the NIH Blood Bank. The procedure is rapid, simple, and safe. A level of approximately 60% Hb A can be achieved after a 5 unit exchange in most patients.

2. Standard Ashby immunologic techniques are used to separate recipient and donor red cells after transfusion to quantitate each fraction.

3. Graded bicycle ergometry testing is carried out at one minute intervals with monitoring of heart rate and arterial blood lactate concentrations to define the anaerobic threshold.

4. An apparatus has been developed to measure, on a breath-by-breath basis, the expired CO_2 and O_2. The ratio of the two, CO_2/O_2, defines the point when lactate begins to accumulate in the blood. Thus, the anaerobic threshold can now be detected in a completely non-invasive manner.

Major Findings:

1. The use of the Hemonetics blood cell separator is an efficient way of carrying out a partial exchange transfusion and we have demonstrated that it is well tolerated and safe. Other investigators have suggested that a sudden increase in oxygen affinity in the patient could have deleterious effects. We have shown that this is not the case.

2. Twelve patients were studied in the initial part of the studies. In each case, exercise performance improved after exchange transfusion. In a total of 20 patients studied so far, 4 have had clinically important transfusion reactions. This has prompted us to the view that exchange transfusion in sickle cell anemia should be reserved for only the most serious indications.

3. A survey of approximately 20 sickle cell anemia patients has been undertaken using the new, non-invasive technique for exercise testing. The preliminary results indicate that there is a large variability in exercise performance in these patients. Nevertheless, most of the patients have anaerobic thresholds higher than would be predicted on the basis of their hemoglobin levels.

Significance of Biomedical Research and the Program of the Institute:

The above findings indicate that considerable improvement in oxygen transport can be achieved in sickle cell anemia with partial exchange transfusion and that the complications which have been suggested from increased oxygen affinity do not occur. Many investigators have raised the question in the past whether treatment of sickle cell blood by pharmacologic means to increase oxygen affinity might have deleterious effects on peripheral oxygen transport. We have demonstrated that this need not be the case and that the improved flow properties of such cells may very well offset any deleterious effects of increased blood oxygen affinity.

Proposed Course of Project:

Because of the complications which have occurred following exchange transfusions, this modality of treatment will not be investigated further. The role of exercise testing with the measurement of anaerobic threshold or assessing disease severity and therapeutic modalities in sickle cell anemia patients will continue. We hope to define correlation between hemoglobin level, blood p50, cardiac function, and anaerobic threshold.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02302-03 CHB

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Purification of Erythropoietin

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

PI:	Neal S. Young	Senior Investigator	CHB NHLBI
Other:	D. Vembu	Staff Fellow	LMH NHLBI
	L. Lee	Medical Technologist	CHB NHLBI
	W. F. Anderson	Lab Chief	LMH NHLBI
	A. W. Nienhuis	Branch Chief	CHB NHLBI

COOPERATING UNITS (if any)

Laboratory of Molecular Hematology

LAB/BRANCH

Clinical Hematoloby Branch

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland - 20205

TOTAL MANYEARS: 0.75	PROFESSIONAL: 0.75	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)

Because of erythropoietin's central role in red cell formation and its possible role in regulation of hemoglobin synthesis, we have embarked on a program to attempt its purification and also to obtain specific antibodies to this substance. In addition, we hope to use similar techniques to purify and obtain antibodies to other growth factors important during erythropoiesis. To date, partial purification of erythropoietin has been accomplished using a wheat germ agglutinin affinity column and this preparation has been used to immunize mice. Spleen cells from hyperimmunized animals will be fused to myeloma cells to form hybridoma cells, some of which may produce monospecific antibodies to erythropoietin. An assay to detect these antibody producing clones of hybridoma cells has been developed. Removal of erythropoietin present in culture media, by antibody bound to an insoluble matrix, is detected by subsequent lack of erythroid colony growth in the culture media.

1132

Objectives: This project has two general objectives. First, we hope to obtain erythropoietin in a pure or nearly pure form. Second, and more importantly, we hope to obtain monospecific antibodies to erythropoietin which can be used to inactivate the hormone in vitro and perhaps even in vivo. Utilizing these antibodies, the precise role of erythropoietin in formation of various types of stem cells may be elucidated. Other growth factors are clearly important during erythroid stem cell growth. One of these, burst promoting activity has been partially characterized by other workers. Antibodies specific to such growth factors can elucidate their roles in erythropoiesis. We hope to use hybridoma technology for purification and identification of antibodies to growth factors such as burst promoting activity.

Methods and Results

1. A 20 to 50 fold purification of erythropoietin has been achieved by the wheat germ agglutinin affinity column technique. This erythropoietin has been used to immunize rabbits. Antisera which inhibits the action of erythropoietin in supporting erythroid colony growth in vitro has been obtained.

2. To establish the hybridoma technology in our laboratory, human hemoglobins A and F are being used as test substances. These are available in large quantities and in pure form. Animals have been immunized and optimal conditions sought for formation of hybridoma cells. A screening assay suitable for detection of antibodies specific for these hemoglobins has been devised.

3. Antibodies with high specificity to a protein may be useful in its purification using affinity chromatography methods. However, erythropoietin is available mainly in impure form; most preparations containing a large number of contaminants. Use of hybridomas to produce antibodies circumvents this difficulty because of the primary cloning of antibody-producing cells. However, a large number of such cell clones must be screened to detect specific antibodies of interest. For antigens available in pure form, such as the human hemoglobins, screening methods have been devised which directly assay for binding of hybridoma antibody (cell supernatants) to antigen immobilized on plastic plates. In the case of the erythropoietin, advantage has been taken of the unique functional property of this hormone, the absolute growth requirement of erythroid cells for erythropoietin in culture. Antisera raised against impure erythropoietin in burro and rabbits is capable of inhibiting erythroid cell growth. However, monospecific antibodies may not inactivate erythropoietin in solution. Therefore, an indirect method of assaying hybridoma supernatants has been devised. Microtiter plates are coated with protein A, which binds the Fc portion of the immunoglobulin molecule. After washing, supernatants obtained from hybridoma clones are added to these wells and allowed to bind to the protein A. Following a second set of washes, limiting quantities of erythropoietin are added to each well. In the presence of antibody to erythropoietin the hormone is removed from solution. The solutions are then transferred to mouse spleen cell cultures. Erythroid cell colonies are assessed two days later. The absence of erythroid cell growth indicates the presence of anti-erythropoietin antibodies immobilized on the protein

A-coated plate. We have demonstrated that this method is as effective as direct addition of antisera to cell cultures using heterogeneous antisera prepared against erythropoietin in burros. This system will be employed in detection of mouse hybridoma antibody.

Significance to Biomedical Research in the Institute Program: A clear understanding of erythropoietin's role may provide insight into the mechanism of hemoglobin switching. One of the major goals of the work in this Branch is to devise techniques suitable for inducing hemoglobin F synthesis in patients with severe hemoglobinopathies. This project is part of that general overall goal. Furthermore, the understanding of normal physiological processes which occur during formation of red cells may provide insight into the diseases in which red cell formation is deficient, namely aplastic anemia and pure red cell aplasia.

Proposed Course of Project:

We hope in the next several months to obtain antibodies specific for hemoglobins A and F using the hybridoma technology. Once that we are confident that we can use hybridoma cells for making antibodies to specific proteins we will immediately proceed to apply similar technology to erythropoietin and burst promoting activity.

Publications:

NONE

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-02303-02

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Oxygen Transport in High Altitude Natives

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

PI:			
R.M. Winslow	Senior Investigator	CHB	NHLBI
Other:			
C. Monge	Prof. Med. Universidad Peruana Cayetano Heredia, Lima, Peru		
N.J. Statham	Biologist	CHB	NHLBI
C.C. Gibson	Engineer	DRS	BEIB
E. Dixon	Guest Worker	CHB	NHLBI

COOPERATING UNITS (if any) Division of Research Services, Biomedical Engineering and Instrumentation Branch, Universidad Peruana, Cayetano Heredia, Lima, Peru, and Johns Hopkins University, Baltimore, Maryland.

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/2	1/2	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

This project is part of an overall effort to understand the role of the blood in the transport of oxygen from air to tissues. High altitude natives are particularly suited to this study because the lack of oxygen and resulting polycythemia present an ideal setting for the study of the interaction of the variables involved in oxygen delivery. The results are directly applicable to sea level man with respiratory and cardiac disease and anemia.

1135

PHS-6040
(Rev. 10-76)

Methods:

1. Whole blood oxygen affinity is measured using methods developed at the NIH in conjunction with LT, BEIB, NIH, and the University of Milan.

2. Incremental bicycle ergometry testing is employed using a simple method of collecting expired gas and analyzing its volume and oxygen content. In selected patients, arterial blood lactate is measured as a function of exercise intensity.

3. Measurements of blood viscosity and flow are made using standard viscometers and nucleopore filtration.

Findings:

1. The presence of polycythemia introduces a significant error in the measurement of blood pH. This is presumed to be due to the interaction of red cells and the glass pH electrode. An empirical correction curve was developed to obtain plasma pH from blood pH measurements.

2. Natives of Morococha, Peru (4,532 M, barometer pressure = 435 mm Hg) had slightly elevated plasma pH, and reduced arterial pCO_2, representing a barely compensated respiratory alkalosis.

3. A wide variability of 2,3-DPG concentration and whole blood p50 was found with no obvious clinical or hereditary explanation.

4. A sub-population of patients with excessive polycythemia was identified in Cerro de Pasco, Peru (4,200 M, barometric pressure 455mm Hg). Their hematocrits ranged from 65 to 75 percent. Studies of pulmonary function in these subjects revealed that they had a restrictive pattern of lung disease, arterial hypoxia, elevated 2,3-DPG and p50, and compromised exercise performance compared to controls. Clear improvement in exercise performance can be achieved in these subjects by phlebotomy.

5. The development of polycythemia at high altitude cannot be ascribed to a single stimulus such as hypoxia. In addition, industrial exposure, and possible genetic differences between individuals may also contribute.

Significance to Biomedical Research and the Program of the Institute:

Oxygen delivery to tissues is a complex relationship between pulmonary function, blood oxygen affinity, hemoglobin concentration, blood flow, capillary density and mitochondrial activity and efficiency. These same factors determine O_2 delivery in sea level patients with various diseases. High altitude natives provide experiments of nature in which the balance among these factors can be studied.

We conclude that man is not truly adapted to high altitude residence (4,530 M). Comparing our results with other results in the world literature,

it seems that man does successfully adapt to life at altitude between 3000-3500M. At those altitudes oxygen affinity and 2,3-DPG concentration are normal and hemoglobin concentration is slightly increased. We believe that as hypoxia progresses the ability of man to adapt diminishes and disease can result when these mechanisms are overtaxed.

The development of excessive polycythemia in high altitude natives is an over response to hypoxia. In the adult, abnormal pulmonary function, right ventricular hypertrophy and pulmonary hypertension all result from life-long hypoxia. As hematocrit rises, blood viscosity rises exponentially. This high viscosity reduces blood flow and oxygen delivery to tissues. The present studies represent an approach toward determining the ideal combination of hematocrit and blood flow properties for individuals in an effort to maximize their performance. Since the major problem in sickle cell anemia is also impaired capillary blood flow, the results are directly applicable to that disease as well.

Proposed Course of the Project:

This NHLBI project will be terminated on August 30, 1979, because Dr. Winslow is leaving the NIH to assume a position at the Center for Disease Control in Atlanta, Georgia.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-02304-02

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Regulation of Erythroid Cell Growth in Culture

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

PI:

N.S. Young	Visiting Expert	CHB	NHLBI

Others:

L. Lee	Biologist	CHB	NHLBI
A.W. Nienhuis	Branch Chief	CHB	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	.25	.75

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A reliable system for the growth and quantitation of erythroid cells obtained from human peripheral blood and bone marrow and murine spleen and bone marrow has been established. This culture system has been employed in three areas. First, it provides means to follow patients with immune suppression of bone marrow on treatment, including plasmapheresis and chemotherapy (See clinical protocol 79-H-90). Second, erythroid cell culture has served as the basis for an assay system to detect antibodies which may functionally inhibit erythropoietin activity (see project #201 HL 02302-02 CHB). Finally, the cell culture system is of intrinsic interest because of its relevance to questions concerning the regulation of globin gene expression.

1138

PHS-6040
(Rev. 10-76)

Methods and Results:

Erythroid cell precursors are cultured in 0.8% methycellulose containing 1% bovine serum albumin, 30% fetal calf serum, and erythropoietin in alpha medium. Several batches of fetal calf serum have been screened in order to provide optimal conditions for erythroid cell growth, especially of the earliest progenitor cell, the burst forming unit (BFU-E). Under our conditions spleen cells obtained from phenylhydrazine injected mice yield approximately 1,000 colony forming units (CFU-E)/10^5 nucleated cells. CFU-E growth varies in a linear fashion with erythropoietin concentration while BFU-E growth has been found to vary sigmoidally, suggestive of a regulatory role for cell-cell interaction. In both instances, erythroid cell growth is highly dependent on erythropoietin concentration.

Peripheral blood and bone marrow samples have also been obtained from human normal volunteers and patients with a variety of hematologic disorders. Variability of erythroid growth from human subjects is considerable. Consistently, however, patients with pure red cell aplasia and aplastic anemia have demonstrated dramatically decreased numbers of erythroid precursors, and several patients have been identified with serum inhibitors of normal erythroid growth under conditions in which erythroid progenitors are briefly exposed to serum or concentrated immunoglobulin in the presence of complement. In contrast, allo-antibodies such as those present in the serum of frequently transfused patients, are not inhibitory to erythroid colony growth from normal bone marrow or peripheral blood. In patients with serum inhibitors undergoing plasmapheresis, erythroid cell culture of peripheral blood is serving as a useful indicator of stem cell quantity, and the potency of serum inhibitors may be titered serially.

The utilization of erythroid cell culture for the assay of antibodies to erythropoietin obtained using hybridoma technology is described in report (201-HL-02302-02 CHB). This method involves a modification of the usual cell culture technique in that cells are plated at high density in microtiter wells containing approximately 0.1 ml tissue culture media, and erythropoietin in culture medium is exposed to plastic plates coated with antibody before being layered over the culture media containing the erythroid stem cells. The presence or absence of erythroid cell growth is a qualitative indicator of the removal of erythropoietin by absorption to specific antibody.

Of the components of the erythroid cell culture system, fetal calf serum is the most complex and least, well-defined. Fetal calf serum may be replaced by feric chloride complexed to transferrin, sodium selenite, cholesterol and lectin for the growth of CFU-E. However, the earlier progenitor, the BFU-E, does not grow with limiting fetal calf serum concentration even with these additives. We have been able to replace the fetal calf serum requirement of BFU-E growth with conditioned media obtained from human spleen. Eight spleens obtained at operation have been minced, triturated, and grown in the absence of fetal calf serum in liquid culture containing tetanus toxoid for 3-5 days. The culture media has been concentrated and absorbed

to concanavalin-A- Sepharose and eluted with a specific sugar. Concanavalin-A- may be removed by absorption to Sephadex. The putative burst promoting activity from normal spleen enhances BFU-E growth and may replace fetal calf serum.

Proposed Course of the Project and Significance to Biomedical Research:

Erythroid cell culture of bone marrow and peripheral blood will be used for periodic assessment of bone marrow disease in a clinical protocol which involves treatment of approximately 40 patients with plasmapheresis and immunotherapy. The effect of drugs, especially androgen preparations, on in vitro bone marrow growth will be compared to the efficacy of these drugs in individual patients.

The erythroid cell culture is also being used as an assay method for detection of antibodies to erythropoietin produced by fused hybridoma cells. The effects of manipulation of cell culture on globin gene expression will be examined utilizing our sensitive radioimmunoassay for hemoglobins A and F. Among the proposed focuses of this research are the effects of variable quantity and different lots of fetal calf serum on hemoglobin F production in burst colonies, the effect of added erythrocytes and erythrocyte membrane as chalones and immunofluorescent examination of cytochalsin blocked multinucleate erythroid cells.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-02305-01 CHB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Calcium Transport in Sickle Cells

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

PI:	Earl Dixon	MARC Fellow	CHB	NHLBI
Others:	Robert Winslow	Senior Investigator	CHB	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Erythrocyte-bound (Ca^{2+} - Mg^{2+})-ATPase has been implicated in the abnormal accumulation of calcium in sickle cells. In this work, the enzyme and its stimulator, calmodulin, have been partially purified from SS, AS, and AA cells in an attempt to determine the specific site of the defect. The results indicate that calmodulin, regardless of the cell type of origin, can stimulate (CA^{+2} - Mg^{+2})-ATPase from AA cells, but the enzyme from SS cells has reduced activity which is not significantly stimulated by calmodulin.

1141

Methods:

Blood from AA, SS and AS volunteers was obtained and membrane-bound (Ca^{2+} - Mg^{2+})-ATPase and its activator, calmodulin were partially purified according to published procedures. Specific activity of the ATPase was measured by the release of inorganic phosphate from ATP at 37^{o} for 1 hour in the presence and absence of calmodulin.

Findings:

1. The specific activity of (Ca^{2+} - Mg^{2+})-ATPase from SS cells is reduced in the presence of calmodulin from AA, AS, or SS cells.

2. The specific activity of enzyme isolated from AS cells is similarly reduced under similar conditions.

3. The specific activity of enzyme isolated from a sickle cell patient post-exchange transfusion is reduced in proportion of the fraction of SS cells.

Significance:

Sickle cell anemia is a debilitating genetic blood disease whose manifestations arise from a mutation of a single amino acid. Although the molecular basis of this disease has been understood for 22 years, the pathogenesis still remains obscured. The remarkable accumulation of calcium within these cells is associated with stiffening and unfavorable rheological properties. It is this stiffening which gives rise to vaso-occlusion and the clinical symptoms of sickle cell disease. It is vital to understand how a point mutation in the hemoglobin molecule can be translated into a membrane alteration. The present studies represent a step in this direction. They suggest that a specific protein, (Ca^{2} - Mg^{2})-ATPase is abnormal in sickle cells and we presume this abnormality results from hemoglobin-membrane interaction.

Proposed Care:

This study will no longer be conducted at the N.I.H. because Dr. Dixon who is on leave from Tuskegee Institute will return to his former position there and Dr. Winslow will leave the N.I.H. on September 1, 1979.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-02306-01

PERIOD COVERED
October 1, 1978 through September 30, 1979

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:

P. Kretschmer	Visiting Expert	LMH	NHLBI
W.F. Anderson	Laboratory Chief	LMH	NHLBI
A.W. Nienhuis	Branch Chief	CHB	NHLBI

COOPERATING UNITS (if any)
Laboratory of Molecular Hematology

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.75	0.75	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Mouse fibroblasts transformed with the thymidine kinase gene derived from herpes simplex virus acquire the metabolic properties which allow them to grow in selective media if the thymidine kinase gene is integrated. Co-transformation of these cells with the thymidine kinase gene in addition to a sample of DNA which includes a globin gene may result in stable integration of both genes allowing the expression of the globin genes to be studied. We have obtained eleven stable cell lines by co-transformation of mouse fibroblasts with the thymidine kinase gene and a DNA segment which includes the human delta and beta globin genes. If the human globin genes are expressed in these transformed cells, we anticipate using this system to characterize the mechanism of their regulation, and using analogous transformation experiments, to explore the molecular defects that affect the β globin gene in patients with β thalassemia.

1143

Objectives:

Due to the development of DNA splitting and gene cloning techniques, certain single specific genes with defined sequences other than those of viral origin can now be obtained in a homogeneous state and in large quantity. Consequently, it is possible to study the organization of such genes by restriction mapping and to depict the primary sequence of these genes 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. Recently, it was found by Axel and his colleagues that transformation of recipient cells with Herpes TK gene in the presence of a second extraneous gene usually results in the incorporation of both genes. Thus non-selectable genes can be introduced into cells even without the ligation of the second gene to the selectable marker gene. The cells selected from co-transformants contain the non-selectable gene, the expression of which can then be studied.

These 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 studied by examining the RNA transcripts and globins synthesized. Data obtained from the expression of normal globin genes, truncated globin genes, naturally occurring mutant globin genes (e.g. thalassemia DNA) and mutagenized globin genes can be correlated with DNA sequences to identify the functional role of each part of the globin genes. Globin genes introduced into non-erythroid cells can also be transferred to mouse erythroleukemic cells by somatic cell hybridization. Such studies may be useful in searching for cellular factors which interact with specific regions of globin genes to achieve differential and coordinated expression.

Methods:

1. Preparation of transforming DNA: A purified 3.4 kb fragment containing the Herpes TK gene was cloned using the plasmid pBR322, into E. coli with the help of L. Enquist. Plasmid DNA was prepared using cleared lysate Ethidium Bromide - CsCl equilibrium centrifugation. The human delta and beta globin genes cloned into the vector Charon 4A (HβG-1) was obtained from T. Maniatis and a 4.4 kb fragment containing only the β globin gene was also subcloned using pBR322. HβG-1 DNA was prepared from purified bacteriophage by standard techniques.

2. DNA tranformation: Herpes TK gene and HβG-1 (containing the human delta and beta globin genes) were co-precipitated with calcium chloride and used to transform L-cells. Transformants were selected in HAT medium.

3. Cloning of transformed cell lines: Two weeks after DNA treatment, transformed cells appeard as individual clusters of cells on the petri dish. The cells were cloned using the cylinder cell cloning technique. Early passage cells are frozen away as stock reserves.

4. Identification of globin genes in transformed cell lines: High molecular weight DNA is prepared from each transformed cell clone using Blin's procedure, cut with suitable restriction endonucleases, electrophoresed in agarose gels followed by transfer of the DNA to nitrocellulose filter papers using Southern's blotting technique. The filter papers are then hybridized with nick-translated human globin gene probes.

5. RNA preparation: RNA from transformed cell lines containing human globin genes will be prepared using Guanadinium-HCl-CsCl equilibrium centrifugation technique or the hot phenol - SDS method.

Major Findings:

1. Circular and linearized plasmid DNA transforms L-cells with comparable efficiency at approximately 1 transformant/20 ng of DNA/10^6 cells. In the absence of carrier DNA, transformation does not occur.

2. Out of the original twenty transformants isolated after co-transformation with HβB-1 DNA and the Herpes TK gene, eleven eventually survived in HAT medium. Several transformed cell lines have distinctly different cell morphology and cell size compared to the original non-transformed L-cells. Growth rates of these transformants are also variable, ranging from a generation time of 20 hours similar to the original L-cells, to about 30-40 hour generation time.

Significance to Biomedical Research and the Institute Program:

These studies may provide a means of identifying the genetic basis of human thalassemia by studying defective human globin gene expression in cultured cells. This may result in individually designed therapy for each patient according to their specific type of globin gene lesion, as well as to provide a model of human gene regulation.

Proposed Course of Project:

During the next few months, it should be possible to quantitate the human globin gene copy number and to identify the size of the human DNA fragments in each transformed cell line by restriction endonuclease analysis and hybridization. RNA extracted from cells containing human globin genes will be examined for human globin mRNA. RNA processing will be studied by examining the precursors -mRNA relationship using the Northern technique followed by hybridization. The synthesis of human globin will be studied by *in vitro* translation and by immunoprecipitation with human globin chain specific antibody. Cloned normal human globin gene DNA will be manipulated *in vitro*, used for transformation, and the expression of the altered genes

in the cultured cells will be studied. Once human thalassemic genes are cloned, their expression will be studied accordingly. In collaboration with the Molecular Hematology Laboratory, transformation by microinjecting the TK and globin genes into MEL cells is also being explored.

Publications:

None.