# ANNUAL REPORT
# OF
# PROGRAM ACTIVITIES
## NATIONAL HEART AND LUNG INSTITUTE
## Fiscal Year 1975

U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
Public Health Service National Institutes of Health

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## Fiscal Year 1975

NATIONAL INSTITUTES OF HEALTH
NATIONAL HEART AND LUNG INSTITUTE
Annual Report
July 1, 1974 - June 30, 1975
Office of the Director

The mission of the National Heart and Lung Institute is 1) to conduct and support research on the heart, blood vessels, blood, and lungs and on their diseases; 2) to develop and evaluate means of prevention, diagnosis, and treatment and to encourage application of proven techniques by the medical community; and 3) to provide support for the training of new research workers, clinical scientists, and teachers in the cardiovascular and pulmonary disease fields. Some of the Institute's activities in key program areas during fiscal year 1975 are briefly highlighted below.

## Heart and Vascular Diseases

A major concern of the Institute has been the support of clinical trials to determine whether and to what extent illness and death from coronary heart disease and other cardiovascular diseases can be reduced by timely interventions against major risk factors. Studies recently completed or in progress include:

--The Coronary Drug Project, begun some 8 years ago and completed this fiscal year, reveals that lipid-lowering drugs are not of value in improving long-term survival among patients who have already sustained one or more heart attacks.

--The Multiple Risk Factor Intervention Trial (MRFIT) will test the hypothesis that reducing blood cholesterol levels, reducing elevated blood pressure, and reducing or eliminating cigarette smoking may reduce morbidity and mortality from coronary heart disease among men at increased risk because of the presence of some combination of these factors. Recruitment of the 12,000 volunteers needed for the study is well along at the 20 participating centers and should be completed by January, 1976.

--The Hypertension Detection and Followup Program involves 14 participating centers and more than 11,000 patients with high blood pressure in a 5-year study to assess the effectiveness of adequate blood-pressure control in reducing morbidity and mortality from cardiovascular diseases.

--The Coronary Primary Prevention Trial, underway at 12 Lipid Research Clinics, will evaluate the preventive value of lipid-lowering diets and drugs in 4,000 men who have elevated blood lipids, but have not experienced a heart attack.

—Recruitment of patients began in May for the Aspirin-Myocardial Infarction Study (AMIS) to determine whether aspirin, which inhibits the aggregation of blood platelets, will reduce the threat of recurrent heart attacks and heart attack deaths among persons who have had at least one such attack. The study will involve 30 participating centers, 4,000 subjects, and will run for three years.

Another clinical trial, scheduled to begin in the near future at 12 clinical centers, will compare coronary artery bypass graft surgery with non-surgical medical management of coronary artery disease to determine the clinical conditions under which such surgery is appropriate and most likely to yield good results.

Other activities in the heart and vascular disease fields included 1) establishment of 9 Specialized Centers of Research for basic and clinical studies on acute and chronic ischemic heart disease; 2) continued support of research on means of protecting ischemic heart muscle, minimizing permanent heart damage resulting from acute heart attacks, and accurately assessing the extent of that damage; 3) continued development of non-invasive instrumentation for the detection and evaluation of cardiovascular diseases in symptomatic and asymptomatic patients; and 4) research concerned with the causes, prevention, and emergency treatment of sudden cardiac death.

## Lung Diseases

The lung contains approximately 40 different types of cells, each with some specific function that may be altered in disease. Recent developments in cell-culture and separation techniques show promise of permitting detailed *in vitro* studies of various cell types, including their response to various agents and insults that may operate in the development of lung disorders. Receiving special emphasis has been research on the cells of lung connective tissue and their synthesis of the structural proteins collagen and elastin, and research on the Type II cell, which plays a role in lung repair and also secretes the surfactant needed to prevent collapse of the air-sacs, or alveoli, of the lung. Other basic studies planned or in progress are concerned with respiratory mucin, animal models of pulmonary fibrosis, and the development of markers for various types of lung cells.

Hyaline membrane disease, or neonatal respiratory distress syndrome, is a major cause of death in the newborn, especially among premature infants. Mortality from this disorder may be dramatically reduced as a result of two recent developments: one is a test involving sampling of the amnionic fluid that can identify the high-risk infant before birth; the other is a safe, effective method of applying continuous positive airway pressure to inflate infants' collapsed lungs and preserve adequate respiratory function.

Support was continued during fiscal 1975 for clinical trials of the extra-corporeal membrane oxygenator in the management of acute respiratory failure at 9 participating institutions. Acute respiratory failure carries a

mortality rate of about 40 percent. It is believed that this rate can be substantially reduced by using oxygenators to provide temporary respiratory support until the patients' lungs can recover sufficiently to resume their respiratory duties.

The Division of Lung Diseases has completed the first phase of its National Pulmonary Faculty Training Program, designed to strengthen pulmonary faculties at schools of medicine or osteopathy that have not yet developed adequate programs in respiratory diseases. Applications will soon be invited from these schools nominating junior faculty members for extensive pulmonary training at medical centers selected for their strong academic programs in these fields.

The Institute also sponsored a number of workshops in pulmonary-disease subject areas. The proceedings of some of these were published for distribution to the scientific and medical communities. Published reports included Hematological Analysis of Extracorporeal Circulation; Lung Metabolism; and Lung Cell Separation, Identification, and Culture.

## Blood Diseases and Blood Resources

Hemophilia remains the prime target of most Institute-supported research on hemorrhagic diseases. Recent research has indicated that most hemophiliacs have an abnormal form of antihemophilic factor (AHF) rather than an absolute deficiency of this clotting factor. The abnormal AHF does not permit adequate blood clotting, however, so that normal AHF must be provided, usually as a concentrate, to prevent or control bleeding episodes. But data from research on this aspect of hemophilia also hold promise of yielding more reliable means for detecting carriers of hemophilia before they give proof of it by bearing hemophilic sons. One promising diagnostic scheme has been developed and is currently being evaluated.

At the other pole from hemorrhagic disorders are clotting complications of heart and blood vessel diseases that are often responsible for their disabling or lethal manifestations. Noninvasive techniques have been developed for diagnosis of thrombotic lesions early in their development. These have been used effectively in recent clinical trials evaluating the effectiveness of anticoagulants and inhibitors of platelet aggregation in the prevention of deep venous thrombosis. Highly effective in some patients, these agents, unfortunately, appeared to work least well in patients undergoing orthopedic surgery, who are particularly susceptible to clotting complications during the postoperative period.

Institute-supported research on sickle-cell anemia has included clinical trials of anti-sickling agents for the prevention or relief of sickle cell crises. A clinical trial of oral cyanate was discontinued when the drug was found associated with peripheral neural disturbances and the development of abnormal reflexes in some patients.

Research in Cooley's anemia ranges from studies of the faulty hemoglobin-synthesizing machinery of red cells from patients suffering from the disease to the quest for effective chelating agents to treat the iron overload that often develops as a consequence of repeated transfusions required by these patients.

Other studies are seeking inexpensive and effective methods for identifying abnormal hemoglobins during the prenatal period, at birth, or during later life. One recently developed method for identifying hemoglobin $A_2$ may also be of value for detecting a carrier state of Cooley's anemia.

## Intramural Research

Among the findings reported by the Division of Intramural Research during FY 1975 were the following:

--The extent and severity of heart-muscle damage resulting from acute heart attack may be a critical factor affecting survival and also the amount of residual disability after recovery. NHLI scientists have demonstrated in animals that nitroglycerin, given in combination with methoxamine or phenylephrine, reduced the extent of heart-muscle damage resulting from induced heart attacks and reduced the threat of heart-rhythm disturbances that are a frequent and sometimes lethal consequence of such attacks. This drug regimen is currently being clinically evaluated at NHLI.

--NHLI surgeons report that xenograft heart valves (from pigs), mounted on prosthetic frames for ease of insertion, have out performed artificial disc valves for mitral or tricuspid valve replacement. Recipients of xenograft valves had a much lower mortality rate during the first six months after surgery than did disc-valve recipients. Moreover, during this period the xenograft recipients, though receiving no anticoagulants postoperatively, developed no clotting complications, whereas 30 percent of the disc-valve recipients developed such complications despite maintenance on anticoagulant drugs.

--NHLI scientists have developed methods for casting very thin membranes from silicone rubber that are free of the pinhole defects that have been the chief cause of membrane failure in blood oxygenators. The membranes, incorporated into a spiral coil membrane oxygenator developed at NHLI, have shown excellent gas-exchange and blood-compatibility characteristics during prolonged periods of blood oxygenation in experimental animals (sheep).

--In hemoglobin synthesis, two protein chains--the alpha chain and the beta chain--are combined to form the finished molecule. In Cooley's anemia, however, beta chain synthesis

is abnormally slow; excess alpha chains pile up, then precipitate in the red blood cells, causing them to be destroyed. NHLI studies have shown that the problem in Cooley's anemia is a scarcity of the mRNA that directs the assembly of the beta chain. The betaglobin mRNA is normal, as are the beta chains it produces; but the red cells from victims of Cooley's anemia contain too little of it to keep pace with the normal rate of alpha-chain synthesis.

## National Research and Demonstration Centers Program

During fiscal 1975, the Institute awarded funds for the establishment and support of three National Research and Demonstration Centers under a provision of the National Heart, Blood Vessel, Lung and Blood Act of 1972. The Act authorizes the eventual establishment and support of up to 30 such centers to 1) carry out basic and clinical research on heart and blood vessel diseases, lung diseases, blood diseases or blood resources; 2) provide demonstrations of advanced methods of prevention, diagnosis and treatment; 3) provide a training resource for scientists and physicians concerned with these diseases; and 4) conduct information and education programs for health professionals and the general public.

Of the three centers established thus far, the program of the Baylor Center will focus on heart and blood vessel diseases, particularly arteriosclerosis and its complications. The center established at the University of Vermont will concentrate on lung diseases, with special emphasis on occupational pulmonary disorders resulting from prolonged exposure to harmful dusts and fumes in various industries and occupations. And the center established at the King County Central Blood Bank in Seattle will be concerned mainly with improvement of procedures for the acquisition, processing, storage, distribution, and clinical use of blood and blood products.

## Other Institute Activities

In March, 1975, the Institute completed and forwarded to the President for transmittal to the Congress the second in a series of annual reports required under the National Heart, Blood Vessel, Lung, and Blood Act of 1972. The report highlights activities, progress, and accomplishments during 1974 and outlines plans for the National Heart, Blood Vessel, Lung, and Blood Program over the next five years. A second annual report was also prepared and submitted by the Institute's principal advisory body: the National Heart and Lung Advisory Council.

The Institute also continued its participation in the National High Blood Pressure Education Program (NHBPEP), which was initiated in 1972 and became fully operational in 1973. NHLI was designated the lead coordinating agency for the program, but it is a joint effort involving some 15 federal agencies and over four hundred other participating groups--professional and private,

national and local. Its program, aimed at alerting health professionals and the general public to the dangers of untreated high blood pressure and the beneficial effects of therapy, encompasses a wide range of activities in education, research, planning, and community services.

The activities of NHBPEP continue throughout the year, but usually peak in May, which has been designated National High Blood Pressure Month for two successive years. In conjunction with the National High Blood Pressure Month, 1975, the Institute's High Blood Pressure Information Center distributed to organizations desiring to participate more than 55,000 kits containing education, detection, and treatment guidelines. In the average year, the HBP Information Center receives some 113,000 requests for information and mails out more than 1.9 million publications.

As an indication of the program's effectiveness, the number of patient office visits for high blood pressure remained about the same during the 8 years prior to 1972; this number increased by 15 percent in 1972, 20 percent in 1973, and 30 percent in 1974.

The Institute's Public Inquiries and Reports Branch has worked closely with the HBP Information Center in such matters as the design, production, and staffing of HBP exhibits, production of publications, and also in setting up the highly-successful HBP screening program carried out as a service to NIH employees during the past year. Providing information on high blood pressure is only one function of this Branch, which during FY 1975 handled more than 32,000 inquiries; mailed out more than 1 million publications; oversaw the production and distribution of 59 new publications issued by the Branch or other NHLI divisions, and issued 27 news releases on a variety of program or research topics. In addition, the PIRB has analyzed the trends of incoming public and professional inquiries in order to identify areas requiring additional affirmative action.

The NHLI portion of the U.S.-U.S.S.R. Health Exchange Program made significant progress during fiscal 1975. There was a continued exchange of Soviet and American health researchers as well as productive planning sessions which were convened here or in the Soviet Union. In several areas of the cardiovascular disease program, joint research has moved from the plannning stage to ongoing basic or clinical studies. Areas which have seen particularly successful collaboration include the pathogenesis of arteriosclerosis, myocardial metabolism, and congenital heart disease. In addition, the separate agreement on artificial heart research and development has resulted in an expanding scientific and technological exchange.

ANNUAL REPORT - DIVISION OF HEART AND VASCULAR DISEASES, NHLI

July 1, 1974 - June 30, 1975

## Overview

The Division of Heart and Vascular Diseases (DHVD) is responsible for planning and directing the National Heart and Lung Institute's research grant, contract and training programs in heart and vascular diseases. These programs encompass basic research; targeted research; clinical trials; and education, demonstration and control activities. The Division maintains surveillance over developments in its program areas and assesses the national need for research in the causes, prevention, diagnosis and treatment of cardiovascular diseases and for manpower training in these disease areas. Despite some slight downturn in recent years of the death rate in coronary heart disease, cardiovascular disease continues to be the number one killer in the United States. A major focus of the Division is on arteriosclerosis and hypertension which together account for over 1,000,000 deaths annually. The programs of the Division are broad-based, employing all available funding mechanisms in an attempt to responsibly take three kinds of action: support of new basic research; clinical evaluation of existing basic research findings and concepts through clinical trials; and Education, Demonstration and Control Programs such as the National High Blood Pressure Education Program translating research concepts to practical patient care.

## Administrative Highlights

In September advisory committees for four program areas of the Division were established with new or revised charters. They are: the Cardiac Advisory Committee, the Clinical Applications and Prevention Advisory Committee, the Lipid Metabolism Advisory Committee and the Arteriosclerosis and Hypertension Advisory Committee. Each Committee's purpose is to advise the Director of DHVD on planning, executing and evaluating the Division's programs in the specified area. Three of the Committees held meetings this fiscal year. These committees have been very helpful to the Program Directors and to the Director, DHVD in their discussions and recommendations. The fourth committee, the Arteriosclerosis and Hypertension Advisory Committee will hold its first meeting early in the Fall.

On July 1, 1974 responsibility for the National High Blood Pressure Education Program (NHBPEP) was transferred to the Division from the Office of the Director, NHLI. This organizational transfer, accompanied by a physical transfer of the NHBPEP to the same building housing most other programs of the Division, has resulted in better and closer coordination between all the components of the Division's hypertension program.

Two Branches in the Cardiology Area have been renamed. The name of the Clinical Cardiac Diseases Branch was changed to Cardiac Diseases Branch. The former Cardiovascular Devices Branch is now the Devices and Technology Branch. Concurrent with its change in name, the Devices and Technology Branch became responsible for grants activities in its field, in addition to its previous responsibilities for the research contracts in the Division's Circulatory Assistance program.

Research Highlights

Following are but a few abbreviated examples of the research progress made during the past year:

- The cellular basis of familial hypercholesterolemia (Type II Hyperlipoporteinemia) has been demonstrated. The finding that some cells at least (fibroblasts) in tissue culture have specific receptors for the binding and removal of circulating low density lipoproteins and that these receptors are deficient or lacking in the disorder has opened up a major new area for research and study.

- Progress is being made on developing promising techniques for protecting ischemic myocardium, thus diminishing the amount of heart muscle lost from myocardial infarction. The techniques are in part built upon improved understanding of the basic biochemical metabolism of heart muscle and its derangement when the myocardium becomes ischemic. Experimental therapy includes methods for providing chemical substrates and agents to enhance their entry into cells, methods to improve blood flow in the coronary arteries, and methods to diminish the workload and thus the energy needs of the heart.

- Further progress has been achieved in the development of phonoangiography as a functional non-invasive technique for the detection of arteriosclerosis. In a clinical trial involving 35 patients with carotid artery stenosis, a comparison of the stenotic diameters (0.5mm to 5.0mm) predicted by the non-invasive methods with the diameters estimated by x-ray showed agreement within $\pm$ 0.46mm (S.D.).

- Computer methods to achieve image enhancement and quantification of arterial disease on conventional femoral arteriograms have also been developed which 1) display detected vessel edges; 2) estimate the vessel mid-line and non-diseased lumen; and 3) provide a numerical measure of edge irregularity. A clinical prototype for a stand-alone interactive computer image-processing system is expected to be operational July 1975.

- In the area of hypertension the preliminary finding that patients with low renin hypertension excrete an excess of a novel 16-OH sterol has stimulated several investigators to examine this discovery.

- As one of the few studies providing epidemiological data on women, an analysis of changes in risk factors among Framingham women passing through the menopause has been completed. The most significant finding was that serum cholesterol increases abruptly after menopause. However, this does not account for the 3-4 fold increase in the rate of CHD in the immediate post-menopausal years, which still remains to be "explained".

- The level of alpha-lipoprotein cholesterol in the blood has emerged as a strikingly significant independent discriminant of risk of myocardial infarction within the collaborative studies in Framingham, Albany, Honolulu, Claxton and Israel. Although this association has been reported earlier, its strength of relationship even at ages 65-74 provides a potentially important further refinement of risk assessment both for predictive purposes and for monitoring intervention effects. Prospective data from these studies will become available to confirm this initial cross sectional finding.

- Direct correlation of risk factor levels during life with subsequent severity of atherosclerotic lesions in the coronary arteries at autopsy is being found in the special protocol autopsy studies which are part of the Puerto Rico and Honolulu prospective epidemiological studies. Significant associations of the average percent of intimal surface involved with raised atherosclerotic lesions have been found with preceding levels of serum cholesterol, blood glucose, systolic and diastolic blood pressure. These important findings are beginning to provide a more direct evaluation of risk factor relationships to specific lesions of atherosclerosis as endpoints whereas previous epidemiological data have only related risk factors to clinical events of coronary heart disease.

## Ongoing Research Programs and New Initiatives

- Specialized Centers of Research in Ischemic Heart Disease

  Nine grants have been awarded to investigators in this new network of specialized centers, replacing the Myocardial Infarction Research Units (MIRU's) which pioneered in developing innovative and effective techniques in basic and clinical research and treatment of heart attacks. These new specialized centers have a broader mandate than the MIRU's and are conducting basic and clinical research in both acute and chronic ischemic heart disease.

## Specialized Centers of Research in Hypertension

Five Hypertension SCOR's were established five years ago and have been productive in the study of the etiology and pathogenesis of hypertension and in the development and application of new knowledge essential for the improved diagnosis and management of hypertension. This year a new competition was held for Hypertension SCOR's. Fifteen applications were received and are under review and consideration for funding in FY 1976.

## Specialized Centers of Research in Arteriosclerosis

The thirteen current Arteriosclerosis SCOR grants are due to expire next fiscal year. An evaluation of the current program and activities, by NHLI staff, advisors and SCOR participants, indicated that good progress had been made in the integration of basic and clinical research efforts and in establishing effective multidisciplinary bases for the research programs. Based on this determination that the purposes and approach of this program were valid, an announcement for new open competition for the continuation of this program was issued this fiscal year for funding in FY 1977.

## Protecting Ischemic Myocardium and Minimizing Infarct Size

A program focussed upon protecting ischemic myocardium was established in 1971. The central importance of the problems in this area and the evidence that promising techniques and approaches could be further rapidly developed, led to the release of a competitive RFP last December; of the 57 proposals received, 13 were selected, after review by outside experts and the DHVD staff, for award of contract.

## Quantifying the Size of Infarcted Myocardium

Techniques to quantify infarct size have great usefullness in prognosis as well as in assessing the efficacy of various proposed interventions designed to reduce or limit the size of ischemia, infarction or scarring of the myocardium. A contract program in this area has been conducted since 1971. Review of the needs and progress in this important area led to the decision to continue the program and an RFP was issued last December. Of the 56 proposals received and reviewed by outside experts and the staff of the Division, 9 were selected for award of contract.

## Sudden Cardiac Death

A contract program in this area has been in existence since 1970 and has been very productive. Research in this program is directed toward a better understanding and prevention of sudden death and includes in its scope prophylaxis and early

therapy, evaluation of pathophysiological mechanisms, precipitating factors and recognition of risk factors. Upon careful review of the breadth and scope of the program it was judged more appropriate to move the continuation and expansion of this program to the grant ledger rather than to continue it in the contract area. Applications for this program are currently under review.

- Development of Non-Invasive Diagnostic Instrumentation

In recognition of the pressing need to improve the diagnosis and evaluation of asymptomatic as well as symptomatic patients and to enhance the effective evaluation of current treatment modalities the Division issued an RFP for the development of noninvasive diagnostic instrumentation. Of the 34 proposals received in response to the solicitation, 8 have been selected for funding this year and a ninth is under consideration for funding next fiscal year.

- Non-Human Primate Models of Arteriosclerosis, Hypertension and Dyslipoproteinemia

A need of high priority has been the development of resources of appropriate models in nonhuman primates to facilitate research into the chronic development and regression of lesions and into the pathophysiology of arteriosclerosis, hypertension and dyslipoproteinemia. To meet that need the Division issued an RFP for the development and supply of suitable animals. Of the sixteen proposals received, six have been selected for funding this fiscal year.

- National Cardiovascular Research and Demonstration Center in Houston

The National Research and Demonstration Center for Cardiovascular Diseases was formally dedicated at Baylor University in Houston, Texas in March. This, the first such Center in Cardiovascular Diseases authorized under the Heart and Lung Act of 1972, has a well rounded and integrated program of basic and clinical research, education and demonstration projects. This new Center holds great promise of being in itself a model and demonstration of the validity of the concept that both basic research and application of research results have something to gain from being in close proximity, and that the gap between the bench and the bedside can be narrowed. An important and innovative ingredient built into the program of the NRDC is a comprehensive evaluation plan under which all activities of the Center will undergo periodic and searching evaluation, by the Center itself, by the NHLI and by the scientific and medical communities.

- National High Blood Pressure Education Research Program

The purpose of this program, now in its second year, is to foster efforts that will result in a better understanding of the characteristics of an individual that may relate to adherence behavior and the education interventions that will enhance a patient's initial and long-term adherence to hypertensive therapy. Six grants were awarded last year, and this year, in response to the second solicitation, an additional five grants were awarded.

- Nutrition Research and Education

Good progress continues to be made in the area of nutrition research and education. Among the activities of this fiscal year was a Nutrition-Behavior Conference which brought together the investigators involved in nutrition studies from the Lipid Research Clinics, Multiple Risk Factor Intervention Trial, Specialized Centers of Research Program and the Cardiovascular Research and Demonstration Center to share the experience and results of these studies. The nutrition teaching materials which have been produced within these programs are expected to have wide-spread general value. The common LRC-MRFIT system for processing diet information and calculating nutrient values has been placed in operation at the Nutrition Coding Center at the University of Minnesota.

- Education and Demonstration Programs

Several components of the Division's program in education and demonstration are described elsewhere in this report, namely the National Research and Demonstration Center and the High Blood Pressure Education Research Program. A report on the opportunities and needs in cardiac rehabilitation was prepared and issued by a Task Force established by the Division. As a beginning step in implementation of that report the Division is planning, with the help of expert consultants a baseline survey of knowledge, attitudes and behavior on the part of physicians and their patients vis a vis cardiac rehabilitation. The National High Blood Pressure Education Program continues to make solid progress. Over 100 National, State and local organizations, including voluntary groups, are involved in this activity, under the leadership of the Division. Public awareness is increasing about the importance of regular blood pressure checks and of regular taking of medicine by persons found to be hypertensive. Many communities have launched high blood pressure screening and follow-up programs. Over 300 communities have received assistance and materials from the Division's High Blood Pressure Information Center. In addition the Center has responded to well over a half million requests for information and has developed and is operating an ongoing mass media education campaign which has presented education messages to an estimated 75 percent of the Nation's population. Much remains to be done in this area, however, particularly with respect to increasing adherence to available treatment on the part of persons with high blood pressure.

## Clinical Trials

This year one of the Division's Clinical Trials came to its scheduled end and another began, bringing to five the number of large scale Clinical Trials the Institute is currently supporting. Brief descriptions of their purpose and status follow.

The Coronary Drug Project ended this year in accordance with its planned schedule. This study, begun over eight years ago, evaluated the efficacy of several lipid lowering drugs in patients who had had at least one heart attack. Two years ago three of the drugs were dropped from the study because they had adverse effects without compensating benefits. Neither of the two drugs that were continued for the entire duration of the study, clofibrate and nicotinic acid, had any effect on mortality. Clofibrate, in fact was associated with a high degree of cardiovascular morbidity and while nicotinic acid decreased angina and new heart attacks it was associated with frequent side effects. These results have been widely circulated in the medical literature. It should be emphasized that these negative findings refer only to patients who have had previous heart attacks, and do not indicate whether the two drugs are useful for persons who have not had any heart attack. Despite the disappointing negative results, the study was extremely worthwhile in two respects: first, it should result in lower costs to patients who will not have these drugs prescribed, and second, the information obtained on the natural history of myocardial disease is very useful.

The Hypertension Detection and Follow-Up Program is a multicenter cooperative effort designed to determine the effectiveness of systematic antihypertensive therapy in reducing clinical morbidity and mortality in persons with elevated blood pressure in fourteen community-based populations. Participants include men and women between the ages of 30-69, many with mild hypertension as well as those with moderate and severe disease, in a cross-section of major ethnic and racial groups and socio-economic strata. More than 11,300 hypertensive participants have been randomized to either stepped care or referred care. "Stepped care" is a series of carefully controlled drug regimens for persons who are assigned to HDFP clinics for their hypertension medication. Persons not selected for "stepped care" are referred to their own physicians for treatment, and are in the "referred care" group. At 12 months after baseline, approximately 76% of the stepped care participants are maintaining active participation and mean diastolic pressure reduction is 13.8 mm Hg. About 50% of the referred care participants are also under therapy and the mean reduction of diastolic blood pressure is 7.3 mm. More energetic efforts will be needed to increase the proportion of the stepped care group who reach goal blood pressure because of the greater than expected treatment effect in the referred care group.

The Multiple Risk Factor Intervention Trial is designed to study whether the reduction of serum cholesterol, reduction of elevated blood pressure and elimination or reduction of cigarette smoking will produce a significant reduction in morbidity and mortality from coronary heart disease. Twenty

clinical centers are each screening 12,000 to 20,000 men aged 35-57 to identify and enroll a total of 12,000 men who are at increased risk of developing coronary heart disease because of combination of these three major risk factors. Over 7,000 of the 12,000 participants needed for the trial have been enrolled so far and the primary recruitment phase of the study is expected to be completed by January 1, 1976. The study period for each person in the Trial is to be six years from enrollment.

The Lipid Research Clinics Coronary Primary Prevention Trial is designed to test the hypothesis that lowering the levels of lipids in the blood (by diet and drugs) will decrease the incidence of heart attacks and heart attack deaths. Twelve clinics are conducting this study, which will involve 4000 male subjects who have high blood levels of lipids but who have not had a heart attack. Over 1200 subjects have already been randomized into the study, and it is expected that recruitment will be completed by January, 1976. The study is scheduled to run for 7 years.

The Coronary Artery Surgery Trial has been in the planning and protocol preparation phase for the past two years and will be entering its full operational phase late this fiscal year. Twelve clinical centers will evaluate coronary artery bypass graft surgery by a series of randomized studies and a patient registry. Over 25,000 such operations are being performed each year. This study hopes to provide careful assessment of the clinical conditions in which such surgery is appropriate and compare the results of surgery with non-surgical medical treatment, both in terms of morbidity and mortality.

The Aspirin-Myocardial Infarction Study was initiated this year. It has been observed in a number of retrospective studies that regular ingestion of aspirin was associated with lower incidence of heart attacks. This study is a carefully designed prospective study of the possible efficacy of aspirin as a preventive for recurrent heart attacks and heart attack death among persons who have had at least one such attack. Thirty clinical centers are participating in this study, which will involve 4000 men and women, each of whom will be followed for at least 3 years.

## Evaluation Studies in Clinical Trials

The size and importance of the Division's clinical trials make it imperative that the Division ensure that they are being conducted in as effective and efficient manner as possible, from both the technical, scientific and the administrative points of view. Accordingly the Division proposed and received authorization from HEW to conduct evaluation studies of two aspects of the trials: the central laboratories that perform the laboratory analysis of blood, urine, lipids, etc., and the central coordinating centers that play a key role in the accumulation, processing and analysis of the large amount of data that are generated in the studies. The evaluation of the central laboratories was started in the Spring. Plans for the evaluation of the coordinating centers are in process and it is expected that an RFP for the conduct of the study

will be issued shortly. These evaluation studies, in which outside experts not directly involved in the operations of the centers will provide advice and recommendations to the Division, will be valuable not only with respect to the current trials but also with respect to future trials in this Division or elsewhere.

Manpower Programs

Since its inception last year, the Manpower Branch has been concerned with the Weinberger Postdoctoral Fellowship Program, Impoundment Restoration Order, Stipend Equalization, National Research Service Award Programs and, finally, the recent administration moratorium on FY 76 Institutional Fellowship Awards. The Division plans to make 84 new Individual Fellowship Awards, 25 new Institutional Fellowship Awards and 23 new Career Development Awards in Fiscal Year 1975. The cardiovascular community, and of even more importance the young potential cardiovascular research scientists, are being confused if not demoralized by the ever changing Federal plans and regulations on fellowship support. It is hoped that some order and stability can be brought into this area in the next year.

ANNUAL REPORT
OF
DIVISION OF BLOOD DISEASES AND RESOURCES, NHLI
July 1, 1974 through June 30, 1975

The programs of the Division of Blood Diseases and Resources seek to improve the diagnosis, prevention, treatment, and cure of diseases of the blood and related disorders, and to improve utilization of the nation's blood resources. These programs encompass basic research, targeted applied research, clinical trials and demonstrations and applications of these research findings in four programmatic areas: bleeding and clotting disorders, sickle cell disease and related disorders, blood resources, and biomaterials research and development. The Division continually assesses the national needs for research in these areas and develops and supports programs to address those needs through the research grants and contracts. Some highlights of accomplishments of the past year are briefly mentioned below.

The Division continues to maintain interest in hemophilia as a major portion of its research in bleeding and clotting disorders. The understanding of the molecular nature of Factor VIII continues to increase. It is now appreciated that most hemophiliacs have an abnormal Factor VIII molecule which does not permit adequate blood clotting. Data derived from research on this aspect of hemophilia has allowed the design of a scheme to diagnose hemophilia carriers. A special workshop was coordinated and sponsored by the Blood Division and the National Hemophilia Foundation to study the adequacy of this method. Hemophilia B, or Factor IX deficiency, must be treated with a plasma fraction different from that used for Hemophilia A, or Factor VIII deficiency. The Factor IX concentrates have been associated with a high risk of thrombotic complications. Recent studies have helped clarify the nature of this problem and led to methods designed to overcome them. A major problem in the care of the Factor VIII deficient hemophiliac has been the spontaneous appearance of inhibitors to Factor VIII. A study of the incidence and clinical importance of these inhibitors has been designed by the Blood Division and will be implemented early in fiscal year 1976. It is hoped that the information obtained will improve treatment of patients with inhibitors and reduce the strain on the blood resource created by these patients.

Studies on the mechanism of thrombosis and means for its prevention and control are being pursued at many levels. There is reason to hope that blood tests will be developed to identify patients with a high risk of thrombotic problems. Non-invasive diagnostic methods, utilizing physical and chemical means, have been developed to diagnose thrombotic lesions in their early stages of development. These methods have been utilized to perform clinical trials which have demonstrated that certain anticoagulant and antiplatelet agents can prevent the formation of deep venous thrombosis and perhaps pulmonary embolism. The status of this field was recently summarized at a workshop sponsored by the Blood Division and the American Heart Association. The information issuing from that workshop, which will

be published and available to the community, will help in the formulation of future research plans and development of educational programs for the biomedical community.

Patients undergoing orthopedic surgery are particularly susceptible to postoperative thromboembolic complications. These patients have shown the least promising results with anticoagulant and antiplatelet agents. The Blood Division is supporting four clinical trials utilizing such agents alone and in combination to clarify the status of this problem. These studies are underway and should be completed within one year.

The Division's interest in Cooley's Anemia (thalassemia) has resulted in the funding of a clinic to test and evaluate new techniques of screening and genetic counseling. Fundamental research is also being funded from which abnormal sub-cellular mechanisms are elucidated in the red cells of thalassemia patients. The problem of iron overload, which develops as a result of chronic transfusions of thalassemia patients, has necessitated a search for iron chelating agents. Presently, a clinical trial of the iron chelator, Desferrioxamine, is under consideration.

As part of the Division research emphasis on sickle cell disease and related disorders, studies of the anti-sickling agent cyanate indicated that this agent, when given by the oral or by the parenteral route, is associated with peripheral neuropathy, characterized by an alteration of the nerve conduction time and the development of abnormal reflexes. These findings have resulted in discontinuance of clinical trials with oral cyanate. In the meantime, extracorporeal studies utilizing cyanate are being continued. A number of other anti-sickling agents are also being investigated. These studies are in the early developmental stage and it is too early to determine possible success of these agents.

Efforts continue to develop more effective and economical procedures to identify abnormal hemoglobins during the prenatal period, at birth and at later times in life. Procedures to identify hemoglobin A, S and F during the prenatal period have been developed and are now being refined. A micro-column chromatography procedure, which was developed a year ago and is presently being field tested, will allow for the identification of hemoglobin $A_2$ which will perhaps make a procedure available to identify a carrier state of Cooley's Anemia.

Recent accomplishments in the development of blood compatible biomaterials include the expanded and improved capability for biological testing and evaluation of candidate materials. Also, a workshop on extracorporeal membrane oxygenators was jointly sponsored and coordinated with the Division of Lung Diseases which reviewed the current state of the art and formulated clinical and research problems on the relation of blood elements to membrane oxygenators.

In the area of blood resources, the Division's efforts have continued to play a catalytic role vis-a-vis the American Blood Commission, initially

in its formation, and presently in the support of its task groups, which will have a major role in providing the data upon which the Commission will implement the goals of the National Blood Policy. Other activities include clinical trials of hepatitis B immune globulin as passive immunization for populations at high risk of contracting hepatitis B. These trials are nearing completion; results will be reported early in FY'76. A prospective epidemiological study of post-transfusion hepatitis has been started to assess, on an ongoing basis, the nature and extent of this problem as we move towards the implementation of an all-volunteer blood donation system. During this year the Division has supported the development of prototype sterile connecting devices between plastic containers. Such devices have many applications in blood banking practice, one of which is the extension of the outdating period for frozen-thawed red cells.

The Division, as a major focus for research and clinical training in hematology and related areas including blood banking sciences, currently supports over 180 trainees. Two coordinated studies are being initiated to assess the further national training needs in these areas.

# DIVISION OF LUNG DISEASES

## ANNUAL REPORT

July 1, 1974 through June 30, 1975

The Division of Lung Diseases plans, directs and evaluates extramural programs addressed to pulmonary diseases and respiratory disorders. Through four Branches (Etiology, Pathophysiology, Special Programs and Resources, and Centers and Control Programs) responsible for both grants and contracts, the Division sponsors programs for (1) Research and Development, (2) Manpower, (3) Research and Demonstration Centers and (4) Prevention, Control and Education. These activities are supported through investigator-initiated research and program project grants, goal-oriented center grants, manpower development awards and targeted research contracts. Every effort is made to achieve a coordinated program and all goal-oriented and targeted programs are evaluated at regular intervals by both the Division's staff and the Pulmonary Diseases Advisory Committee, frequently with the advice of ad hoc consultants. Program evaluations are presented to the National Heart and Lung Advisory Council and Institute staff.

Tangible accomplishments are evident in the Division's effort to interest and involve investigators from basic disciplines, and to foster fundamental studies of the lung in health and disease. Interdisciplinary research with a major emphasis on basic problems is now supported through 17 program project grants, an increase of 42 percent since fiscal 1974. The Young Investigator Pulmonary Research Grant has attracted new investigators to the pulmonary field and it is gratifying to note that this year, as was the case in 1974, approximately half of the new grants are addressed to lung structure or function. National Research Service Institutional Grants and Postdoctoral Fellowship Awards approved this year are also largely concerned with fundamental problems. Contract programs addressed to various aspects of lung structure and function (for example, studies of lung elastin, and separation and culturing of lung cells) have stimulated interest in fundamental approaches that are now being extended, through issuance of requests for contract proposals, to studies of respiratory mucin, pulmonary fibrosis in animal models, and the development of markers for various types of individual lung cells. In addition, the Division continues to support fundamental as well as clinical research through its Specialized Centers of Research, and the investigator-initiated research grant continues to be addressed in large part to basic investigations.

As Specialized Centers of Research (SCOR) grants are approaching the time for competitive renewal, the Division has announced a new Pulmonary SCOR competition that invites new applicants as well as renewal of present active grants. On the basis of four years of experience with this supportive mechanism and consonant with the objectives of the Division's National Plan, this competition requires each SCOR to be limited to one of four major disease categories; namely, Chronic Airways Diseases, Pediatric Pulmonary Diseases, Fibrotic and Immunologic Pulmonary Diseases, and Pulmonary Vascular Diseases. It also requires a clinical emphasis but strongly encourages fundamental investigations relevant to the SCOR goals. The Division believes that the SCOR mechanism

should not duplicate opportunities available through other supportive mechanisms, such as the program project grant. Moreover, institutions with strong programs in more than one disease category are encouraged to submit more than one SCOR application.

The Division's Prevention, Control and Education Program has been initiated with two groups of contracts for educational programs addressed to recognition and early treatment of acute respiratory insufficiency in adults and neonatal respiratory distress syndrome. Other demonstration and education activities are supported through the Vermont Research and Demonstration Lung Center.

Because pulmonary academic manpower is still insufficient to meet national needs, the Division initiated a new program--the National Pulmonary Faculty Training Program--designed to enable institutions with inadequate pulmonary academic staffs to capitalize on resources for excellent training at other institutions. This is a two-phase program. The first phase has been completed with the review of applications from institutions wishing to provide pulmonary training. The Division anticipates (by August 1, 1975) inviting applications from medical schools that wish to nominate junior faculty members who will receive training at one of the approved Training Centers.

The Division is strongly committed to continuing evaluation of its goal-oriented and targeted programs. During the year it has drawn upon ad hoc consultants as well as the Pulmonary Diseases Advisory Committee to assess the Pulmonary SCOR, Pulmonary Academic Award, Contract and Epidemiology Programs as well as a completed group of contracts on Oxygen Toxicity. Review of the Epidemiology Contracts and of the Pulmonary Academic Award Program involved on-site visits. In addition, the Pulmonary Diseases Advisory Committee visited the Vermont Research and Demonstration Center in association with the regular Committee meeting.

To coordinate the different facets of its program the Division sponsors small workshops, which involve SCOR investigators, contractors, grantees and expert consultants, for discussions of issues that are timely and important relative to the Division's National Program. The Division also works with national societies in arranging symposia at their annual meetings. Reports, already issued or in preparation, on workshops held since the last Annual Report are these: *Hematological Analysis of Extracorporeal Membrane Oxygenation, Lung Metabolism,* and *Lung Cell Separation, Identification and Culture.* A symposium sponsored jointly with the American College of Chest Physicians was addressed to the use of extracorporeal membrane oxygenation in acute respiratory failure.

For the information of the biomedical community, the Division distributed reports on its *Contract Program* (updated), *Current Pulmonary Research Programs* (updated), and *Bioengineering Program;* as well as *Procedures for Standardized Measurements of Lung Mechanics and Principles of Body Plethysmography,* and *Report of Conference on Scientific Basis of Respiratory Therapy.*

Finally, the Division held meetings of two steering groups to obtain expert consultation in developing its plans for two small task groups to be initiated in the Fall. One, addressed to Epidemiology of Respiratory Diseases, the other to Prevention, Control and Education in Pulmonary Diseases, will advise the Division on what is known, what is being done, what needs to be done, and how the Division can best contribute to these two important areas.

The Division's major problem is the same as last year; namely, to acquire a professional staff of appropriate background and adequate size to monitor responsibly its complex and growing programs.

NATIONAL HEART AND LUNG INSTITUTE
DIVISION OF EXTRAMURAL AFFAIRS
Annual Report
July 1, 1974 - June 30, 1975

The Division of Extramural Affairs is responsible for advising the Director, NHLI, on research contract, grant, and training program policy. It also represents the Institute on overall NIH extramural and collaborative program policy committees, coordinates such policy within NHLI, and coordinates the Institute's research grant and training programs with the National Heart and Lung Advisory Council. Other major services provided by the Division include: (a) grant and contract management and processing services for the Institute's other divisions, (b) reports and statistics related to the Institute's grant and contract programs, and (c) initial scientific merit review of project grants and research contracts for the Institute.

Increased emphasis was placed upon improved implementation of the Freedom of Information Act and the recently enacted Privacy Act amendments scheduled to become effective later this year. It therefore became essential that the Institute centralize these activities. The Acting Director of the Institute appointed a Freedom of Information Officer in the Division of Extramural Affairs to accomplish this. An index of Institute documents affecting grant and contract programs was developed, and inquiries for technical information are now channeled through this officer.

The Division has continued to serve as the primary liaison to the National Heart and Lung Advisory Council. In collaboration with the Council, several mechanisms were developed which should help the Division to more efficiently carry out its responsibilities. One of these are criteria that define the Program Project Grant as conceptualized and utilized by the NHLI. Guidelines have also been implemented for the individual review of Program Projects by the Council. The Council has asked that criteria for the support of conferences also be developed for their use.

The Division continues to provide broad services to the rest of the Institute. These include:

1. Management requirements for grants and contracts.
2. Central storage and maintenance of official files for all grant programs.
3. Preparation of review materials for Council.
4. Preparation of official and summary minutes of Council actions.
5. Follow-up and close-out for all terminated grants.
6. Operation of the program Policy and Procedures Office.
7. Committee management functions.

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

Clinical Trials
Conference Grants
High Blood Pressure Education Research Program
Hypertension SCOR Grants
Institutional Fellowship Grants
Ischemic Heart Disease SCOR Grants
National Pulmonary Faculty Training Program
Program Project Grants
Pulmonary Academic Awards
Sudden Cardiac Death Research Grants
Thrombosis SCOR Grants
Young Pulmonary Investigator Program

In addition, sixteen contract reviews were held directly related to new RFPs issued by the Institute, and nine renewal contract reviews were held. Thus, a total of 25 review committees were convened to review contracts during Fiscal Year 1975. Furthermore, nine mail reviews related to unsolicited or sole source proposals were conducted by the Review Branch.

In the Reports and Evaluations Branch, the basic NHLI Information System became operational. It consists of several components which may be linked to provide (a) data relating to the management of grants and contracts, (b) data related to research and development activities supported by contracts and grants, as well as (c) data about the major programs and program areas in each division. It is expected that the basic system, which is currently on tape, will soon be converted to an on-line disc. This will shorten response (turn around) time. Overall development of the data system continues with the incorporation of specific items based on the definition of needs by program managers and administrative staff of the Institute. These changes have broadened the capability of the system and have resulted in increased use by program and administrative staff. With basic operations in the Reports and Evaluations Branch finally underway, increased attention will be placed on analytical and evaluative functions in relation to the Institute's overall programs.

The morale of the Division staff has been adversely affected by increased individual workloads coupled with strict hiring limitations. In addition, lack of adequate working space in the Westwood Building has aggravated the morale situation. The lack of sufficient space has resulted in (a) displacement of one supervisor from her office in order that it could be used for temporary storage, (b) conversion of office space for a professional staff member into a storage and processing room during peak workload periods, and (c) continued, and valid, complaints from the Montgomery County Fire Marshal, as well as neighboring organizations, because of cluttered hallways.

The increase in the Institute's programs and the Division's workload has also brought about a significant increase in grant and contract reviews, management procedures, and coordinating activities. Although the Division has continued to meet its deadlines, unless additional positions are made available and our physical space allocation is increased, the real possibility exists that future deadlines may not be met and the morale of the staff will continue to deteriorate.

It is anticipated that next year will bring an increase both in the workload and responsibilities of the Division. We are therefore studying possible internal reorganizations which may increase the efficiency of our use of existing space and personnel. However, without an increase in our current personnel ceiling and space allocation, it is doubtful that we can continue to carry out our responsibilities in the highly professional manner that has been characteristic of this Division.

INTRAMURAL RESEARCH

NATIONAL HEART AND LUNG INSTITUTE

July 1, 1974 - June 30, 1975

INTRAMURAL RESEARCH

Project Reports

ANNUAL REPORT OF THE
LABORATORY OF BIOCHEMICAL GENETICS
NATIONAL HEART AND LUNG INSTITUTE
July 1, 1974 through June 30, 1975

Studies in the Laboratory of Biochemical Genetics focus on defining mechanisms which enable cells to communicate with one another and to respond to hormones and other external influences.

During the past few years, numerous neuroblastoma cell lines were obtained and characterized with respect to neuronal properties such as transmitter synthesis, storage, and catabolism, receptors, and effects of receptor activation.

Additional cell lines with new neural phenotypes were generated and questions of dominance of gene expression and complementation were explored by fusing neuroblastoma with other cells and obtaining hybrid cell lines. The expression of genes for neural properties was found to be dominant with most matings. Some hybrid clones expressed the neural phenotype of the neuroblastoma parent 50 or more cell generations after fusion; many others had specific defects in transmitter synthesis, storage, and catabolism; response to neurotransmitters; action potential reactions; and so forth. Another class of hybrids had acquired new neural properties which were not detected with parental neuroblastoma cells. For example, we previously showed that fusion of mouse neuroblastoma cells with rat glioma cells, both lacking choline acetyltransferase, yields hybrid cell lines with high choline acetyltransferase activity which store acetylcholine and have clear vesicles identical in appearance to those found at synaptic junctions. Additional studies now show that fusion of mouse neuroblastoma cells which lack tyrosine hydroxylase activity with cells from normal sympathetic ganglia from mouse embryos yields hybrid cells with high tyrosine hydroxylase activity that synthesize dopamine, possess muscarinic excitatory acetylcholine receptors, and have both small and large dense-core vesicles. Fusion of neuroblastoma cells with cells from normal retina yielded some cell lines that synthesize catecholamines and another that synthesizes acetylcholine. These results show that fusion of neuroblastoma cells with cells from the normal nervous system generates hybrid cells with new neural properties which have not been detected with the parental neuroblastoma cells. The new neural phenotypes are inherited and thus far have been perpetuated in a fairly stable fashion for more than 100 cell generations. This approach would appear to be a general one that can be used to obtain cell lines with other differentiated properties that can be used to elucidate reactions that are required for cell communication.

Cyclic GMP levels of some neuroblastoma and hybrid cell lines were found to increase up to 200-fold upon activation of muscarinic acetylcholine receptors, resulting in intracellular cGMP concentrations greater than 600 pmoles per mg protein. Both sensitive and insensitive cell lines were found. The cells also have receptors for $PGE_1$ and adenosine which, upon activation, result in rapid elevations of cAMP levels. Thus, the effects of activating pairs of receptors which are functionally coupled to cGMP or cAMP cell responses were studied. The results reveal considerable complexity in the regulation

of receptor mediated events. Activation of the muscarinic acetylcholine receptor elicits both an elevation in cGMP and a decrease in cAMP levels. Conversely, activation of adenosine receptors elevates cAMP and depresses cGMP levels. Unexpectedly, $PGE_1$ was found to increase the concentrations of both cGMP and cAMP. The results suggest that one species of $PGE_1$ receptor affects cAMP levels and another receptor, cGMP levels. Carbamylcholine and $PGE_1$ dependent increases in cGMP are additive; whereas, $PGE_1$ and adenosine dependent increases in cAMP levels are not additive. These results show that informational molecules impinging upon a cell regulate in at least 4 ways cell responses to other species of informational molecules. Current studies focus on defining the mechanisms which underlie the observed phenomena, for similar events may well occur at synapses. The results also show that genes determining receptor species for putative neurotransmitters can be expressed in dividing cells, that the parental programs of gene expression are inherited, and that dividing cells can be programmed with respect to their ability to receive information from different kinds of neurons.

In collaboration with Dr. Werner Klee, various neuroblastoma and hybrid cell lines were assayed for morphine receptors. Cell lines with and without stereospecific, high affinity, morphine receptors were found. Such receptors are particularly abundant in a neuroblastoma x glioma hybrid cell line, for the average hybrid cell possesses approximately $3 \times 10^5$ narcotic receptors. The neuroblastoma parent possesses relatively few narcotic receptors and the glioma parent lacks narcotic receptors. The results suggest that gene expression for opiate receptors may be dominant in the hybrid cell lines studied.

Further studies revealed that morphine inhibits adenylate cyclase activity of cells with morphine receptors but does not affect adenylate cyclase activity of cells without these receptors. Thus, two forms of adenylate cyclase were distinguished; one form sensitive, the other insensitive, to narcotics. Questions pertaining to the mechanism of narcotic addiction and dependence currently are being explored with this system.

$[^{125}I]$-Labeled-α-bungarotoxin has been used as a specific probe for acetylcholine receptors. A highly sensitive histochemical technique for localization of acetylcholine receptors was devised which is based on the binding of bungarotoxin to the receptor followed by complex formation with rabbit antibody against the bungarotoxin. A double antibody step follows involving complex formation with horseradish peroxidase conjugated antibody against rabbit antiserum. Using this procedure, it has been possible to show that mouse diaphragm endplates have a limited distribution of acetylcholine receptors, that denervated muscle shows spreading of receptors and that myasthenia gravis is characterized by the presence of a serum factor that interferes with the binding of toxin to normal muscle endplates.

Some properties of the $Na^+$ influx system associated with the nicotinic acetylcholine receptor ionophore were studied. $Na^+$ uptake behaves cooperatively upon stimulation by cholinergic agonists and activation of $Na^+$ transport exhibits a different temperature profile than the transport process. Activation of the $Na^+$ ionophore by alkaloid neurotoxins is completely inhibited by divalent cations. Other studies indicate that the ionophore is regulated cooperatively by another component that interacts specifically with polypeptide toxins.

A variety of cell types maintained in tissue culture respond to environmental or developmental changes with respect to their complement of ionophores. Inhibition of the growth of neuroblastoma cells by butyric acid or dibutyryl-cAMP results in increased ionophore activity. Embryonic chick skeletal muscle in culture develops $Na^+$ ionophore activity only after fusion of the cells into myotubes. In contrast, stable cultures of rat skeletal muscle have ionophore activity before fusion which is only increased approximately two-fold after fusion. Adult heart cells possess 3 or more types of ionophores, one for $Na^+$, one for $Ca^{++}$ and another for $K^+$. Use of the inhibitor D-600 has shown that embryonic chick heart has a population of ionophores that changes with age.

Induced enzyme synthesis in E. coli requires cAMP. The blockade of such induced enzyme synthesis by glucose or other catabolizable sugars is due to their effects in lowering cAMP levels. We have been exploring the mechanism by which glucose decreases cAMP levels. We have shown that, while glucose does not affect adenylate cyclase in vitro, it effectively inhibits the enzyme in intact cells. The substitution of α-methyl glucoside, a compound that can penetrate cells, but not be metabolized further than the phosphorylated form for glucose as an inhibitor of adenylate cyclase indicates that extensive metabolism of glucose is not required for adenylate cyclase inhibition.

The spectrum of sugars that inhibit E. coli adenylate cyclase varies with the conditions under which the cells have been cultured. A variety of studies indicate that the presence of transport systems for sugars endows E. coli with the capacity to have its adenylate cyclase inhibited by the sugars. These studies suggest that adenylate cyclase in E. coli may be regulated in an inhibitory sense by a mechanism similar to that by which mammalian cell adenylate cyclases are regulated positively by many hormones.

Friend-virus transformed mouse erythroleukemia cells produce hemoglobin on exposure to dimethylsulfoxide and have high levels of acetylcholinesterase. Hybrids formed between these cells and human or mouse fibroblasts were found not to express these differentiated functions and not to produce hemoglobin messenger RNA which suggests that hemoglobin gene transcription is repressed in the hybrid cells.

Fusion of a transformed tissue culture cell line to normal cells which have not been cultured results in hybrid lines in which chromosomes from the normal parent are preferentially lost. This property has been used for gene mapping by tracing the segregation of chromosomes and loss of certain enzymes. We have found that at least 15 isozymes are asyntenic in the mouse. In addition, we observed that genes which are linked in man are on different chromosomes in the mouse, and that some genes that are on the same chromosome arm in the human are linked in the mouse.

Hybrids between human and mouse cells have been analyzed for the expression of oncornavirus. Our results indicate that human genes can regulate the production of the viral RNA-dependent DNA polymerase but do not affect the expression of the viral structural proteins. Human chromosomes 14, 21 and possibly 12 appear to be responsible for this regulation. We also found that human genes can block the induction of type C viral genes from mouse integration sites and can alter the host range of mouse virus.

Project No. Z01 HL 00001-04 LBG
1. Biochemical Genetics
2. Molecular Biology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Acetylcholine Receptors

Previous Serial Number: NHLI-305

Principal Investigators: Mathew Daniels, Ph.D. and Zvi Vogel, Ph.D.

Other Investigators: S. P. Ringel, M.D. (MNB), A. N. Bender, M.D. (MNB), B. W. Festoff, M.D. (MNB), W. K. Engel, M.D. (MNB), Tom Reese, M.D. (LNNS), M. DuBois, M.D. (IDB)

Cooperating Units: NINDS

Project Description:

Objectives: Investigators in this laboratory and others have utilized $^{125}I$ labelled α-bungarotoxin (αBT) as a label for nicotinic acetylcholine receptors in intact and cultured skeletal muscle, and in embryonic and mature retina. The objectives of this study were to devise a histochemical technique of greater sensitivity and resolution for localizing bound αBT and to apply this technique to studying the ultrastructural distribution of acetylcholine receptors in the peripheral and central nervous system during development, in culture, and in the mature state.

Methods Employed: We have employed indirect immunoperoxidase staining of cryostat sectioned, teased, or monolayer cultured materials to which αBT has been bound. These materials are subsequently examined by light and electron microscopy.

Major Findings: We devised a technique of greater sensitivity and resolution utilizing (rabbit) antibody against αBT and horseradish peroxidase-conjugated antibody against rabbit. Using this double immunotechnique, we observed the limited ultrastructural distribution of acetylcholine receptor within mouse diaphragm endplates.

The technique has now found application in three other studies involving acetylcholine receptor distribution on muscle. (1) In human muscle disease involving denervation, the method has been used to detect denervated fibers, in which there is spreading of the receptors. (2) In myasthenia gravis, a muscle weakness disease, the method has been used to detect a serum factor which blocks αBT binding at normal muscle endplates. (3) In cultured embryonic

muscle the method is being used to characterize the ultrastructure of the regions of the muscle membrane containing a high concentration of receptors.

Significance to Biomedical Research: Knowledge of the ultrastructural distribution of acetylcholine receptor is of clear importance in any attempt to understand the role of neurotransmitters and their receptors in the function and development of the nervous system. The α-bungarotoxin-immunoperoxidase technique already has shown promise for the diagnosis and analysis of mechanisms in human neuromuscular disorders.

Proposed Course: We plan to complete the study on cultural skeletal muscle and continue our collaboration in characterizing the myasthenia gravis serum factor. We are also continuing to modify the technique for attempts at ultrastructural visualization of acetylcholine receptors in retina.

Publications:

1. Daniels, M. P. and Vogel, Z.: Immunoperoxidase staining of α-bungarotoxin bound to acetylcholine receptors in mouse motor endplates. J. Cell Biol., 63, 76a, 1974.

2. Daniels, M. P. and Vogel, Z.: Immunoperoxidase staining of α-bungarotoxin binding sites in muscle endplates shows distribution of acetylcholine receptors. Nature, 253, 339-341, 1975.

3. Bender, A. N., Ringel, S. P., Engel, W. K., Daniels, M. P. and Vogel, Z.: Myasthenia Gravis: A serum factor blocking acetylcholine receptors of the human neuromuscular junction. Lancet, March 15, 607-609, 1975.

4. Bender, A. N., Ringel, S. P., Festoff, B. W., Engel, W. K., Vogel, Z. and Daniels, M. P.: Denervated skeletal muscle fibers identified by immunoperoxidase localization of extrajunctional alpha-bungarotoxin binding. Nature, In press.

Project No. Z01 HL 00002-02 LBG
1. Biochemical Genetics
2. Molecular Biology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Morphine Receptors as Regulators of Adenylate Cyclase

Previous Serial Number: NHLI-300

Principal Investigators: Marshall Nirenberg, Ph.D. and Shail Sharma, Ph.D. Guest Worker (F.I.C.)

Other Investigators: Werner Klee, Ph.D. (LGCB)

Cooperating Units: NIMH

Project Description:

Objectives: The objectives are to elucidate molecular mechanisms of narcotic dependence and tolerance, and the effects of narcotics upon the sensitivities of other receptors.

In collaboration with Dr. Werner Klee, various neuroblastoma and hybrid cell lines were assayed for morphine receptors. Cell lines with and without stereospecific, high affinity, morphine receptors were found. Such receptors are particularly abundant in a neuroblastoma x glioma hybrid cell line, for the average hybrid cell possesses approximately $3 \times 10^5$ narcotic receptors. The neuroblastoma parent possesses relatively few narcotic receptors and the glioma parent lacks narcotic receptors. The results suggest that gene expression for opiate receptors may be dominant in the hybrid cell lines studied.

Further studies revealed that morphine inhibits adenylate cyclase activity of cells with morphine receptors but does not affect adenylate cyclase activity of cells without these receptors. Thus, two forms of adenylate cyclase were distinguished; one form sensitive, the other insensitive to narcotics. The interactions between (narcotic receptor) moieties and the adenylate cyclase complex exhibit positive cooperativity; whereas the interactions between narcotic and receptor are not cooperative.

Significance to Biomedical Research: A molecular mechanism for narcotic dependence was proposed wherein the number of adenylate cyclase molecules per cell increases over a period of days when cells are cultured in the presence of narcotics. Cyclic AMP levels then are normal in the presence of a narcotic

inhibitor of adenylate cyclase but are abnormally high in the absence of the narcotic.

Honors and Awards: None

Publications:

1. Klee, W. A. and Nirenberg, M.: A neuroblastoma x glioma hybrid cell line with morphine receptors. Proc. Nat. Acad. Sci., USA, 71, 3474-3477, 1974.

2. Sharma, S. K., Nirenberg, M., and Klee, W. A.: Morphine receptors as regulators of adenylate cyclase activity. Proc. Nat. Acad. Sci., USA, 72, 590-594, 1975.

3. Klee, W. A., Sharma, S. K., and Nirenberg, M.: Opiate receptors as regulators of adenylate cyclase. Life Sciences, In press.

Project No. Z01 HL 00003-02 LBG
1. Biochemical Genetics
2. Molecular Biology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Regulation of Receptor Activity

Previous Serial Number: NHLI-306

Principal Investigators: Hiroshi Matsuzawa, Ph.D. and Marshall Nirenberg, Ph.D.

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: The objective is to define synaptic events using clonal cells as model systems.

Cyclic GMP levels of some neuroblastoma and hybrid cell lines were found to increase up to 200-fold upon activation of muscarinic acetylcholine receptors, resulting in intracellular cGMP concentrations greater than 600 pmoles per mg protein. Both sensitive and insensitive cell lines were found. The cells also have receptors for $PGE_1$ and adenosine which, upon activation, result in rapid elevations of cAMP levels. Thus, the effects of activating one species of receptor upon cell responses mediated by another species of receptor were studied. The results reveal considerable complexity in the regulations of receptor mediated events. Activation of the muscarinic acetylcholine receptor elicits both an elevation in cGMP and a decrease in cAMP levels. Conversely, activation of adenosine receptors elevates cAMP and depresses cGMP levels. Unexpectedly, $PGE_1$ was found to increase the concentrations of both cGMP and cAMP. The results suggest that one species of $PGE_1$ receptor affects cAMP levels and another receptor, cGMP levels. Carbamylcholine and $PGE_1$ dependent increases in cGMP are additive; whereas, $PGE_1$ and adenosine dependent increases in cAMP levels are not additive. These results show that informational molecules impinging upon a cell regulate in at least 4 ways cell responses to other species of informational molecules. The results also show that genes determining receptor species for putative neurotransmitters can be expressed in dividing cells, that the parental programs of gene expression are inherited, and that dividing cells can be programmed with respect to their ability to receive information from different kinds of neurons. Current studies focus on defining the mechanisms which underlie the observed phenomena, for similar events may well occur at synapses.

Publications: None

Project No. Z01 HL 00004-02 LBG
1. Biochemical Genetics
2. Molecular Biology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies of Action Potential and Receptor Ionophores

Previous Serial Number: NHLI-307

Principal Investigator: William A. Catterall, Ph.D.

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: The objectives of this project are (1) to develop biochemical methods for study of action potential and receptor ionophores, (2) to use these methods to study the mechanism of action of receptor and action potential ionophores, and (3) to use these methods to study the regulation and genetic expression of action potential and receptor ionophores in cultured cells.

Methods Employed: Biochemical assays which measure changes in passive $Na^+$ influx were used to study the acetylcholine receptor ionophore and action potential $Na^+$ ionophores.

Major Findings: (1) Studies of $Na^+$ transport by the acetylcholine receptor $Na^+$ ionophore in cultured muscle cells led to the following conclusions: (a) Activation of the nicotinic acetylcholine receptor by cholinergic agonists is cooperative whereas inhibition by antagonists is not. (b) The processes of activation and desensitization are temperature sensitive while the process of ion transport is not. (c) The ionophore functions as an ion channel rather than as an ion carrier. (d) The channel is saturable with $K_M$ for $Na^+$ of 150 mM at 0° and a turnover number of $2\text{-}3 \times 10^7$ ions/min/channel.

(2) Studies of activation of the action potential $Na^+$ ionophore by neurotoxins led to the following conclusions: (a) the alkaloid neurotoxins veratridine, batrachotoxin, and aconitine activate the ionophore by reversible interaction with a single class of sites; (b) divalent cations are competitive inhibitors of the activation by alkaloid neurotoxins; (c) the polypeptide toxins of scorpion venom activate the ionophore by interaction with a different class of sites from the alkaloid toxins; (d) the sites of action of the alkaloid toxins and scorpion toxins are allosterically coupled in a highly

cooperative manner; and (e) tetrodotoxin is a noncompetitive inhibitor ($K_I$ = 8 nM) of activation by neurotoxins. Experiments from other labs suggest that tetrodotoxin acts at the ion transport site for $Na^+$. These results suggest that the activity of the action potential $Na^+$ ionophore is modulated by two regulatory components which bind activating neurotoxins and interact cooperatively in controlling the activity of an ion transport component which binds tetrodotoxin.

Significance to Biomedical Research: The results provide new insights into the mechanism of action and regulation of membrane macromolecules involved in information transfer and processing in the nervous system and in maintenance of normal beating in heart.

Proposed Course: Planned investigations include (1) completing the kinetic analysis of ion transport by the nicotinic acetylcholine receptor of cultured muscle cells (2) initiating studies of ion transport changes associated with activation of muscarinic acetylcholine receptors of neuroblastoma and heart cells, and (3) purifying the active components of the scorpion toxin mixture used in these studies, radioactively labelling, and studying binding by nerve cells and heart cells.

Honors and Awards: None

Publications:

1. Catterall, W. A.: Sodium transport by the acetylcholine receptor of cultured muscle cells. J. Biol. Chem., 250, 1776, 1975.

2. Catterall, W. A.: Activation of the action potential $Na^+$ ionophore of cultured neuroblastoma cells by veratridine and batrachotoxin. J. Biol. Chem., In press.

3. Catterall, W. A.: Cooperative activation of the action potential $Na^+$ ionophore by neurotoxins. Proc. Nat. Acad. Sci., USA, In press.

Project No. Z01 HL 00005-02 LBG
1. Biochemical Genetics
2. Molecular Biology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Biosynthesis of Neurotransmitters in Cell Hybrids

Previous Serial Number: NHLI-303

Principal Investigator: Eliahu Heldman, Ph.D.

Other Investigators: None

Cooperating Units: None

Project Description:

Major Findings: Established cell lines that are able to form synapses in tissue culture may be very useful as a model for investigating the mechanism of cellular communication and synaptic interactions. Cell hybrids between neuroblastoma and normal neuronal tissue may provide such a system since normal neuronal properties that are carried by the hybrids may be potentially important in cell recognition and in their ability to form synapses. The ability to synthesize neurotransmitters and to store them like normal neuronal cells is one of the requirements in this context. Cell hybrids between the established line of neuroblastoma - N18TG2G and rat or mouse retina were tested for their ability to synthesize neurotransmitters. The cells were incubated with radioactive precursors for potential neurotransmitters and the products were extracted, separated by high voltage electrophoresis and identified. Four classes of cell hybrids were observed: (1) Those that accumulated catecholamines. (2) Those that accumulated acetylcholine. (3) Those that accumulated both catecholamines and acetylcholine. (4) Those that did not accumulate any of the possible neurotransmitters tested (catecholamines, acetylcholine, serotonin and GABA). Some of the hybrids were tested for their GABA content and they did show significant amount of that substance. The precursor for the GABA was not glutamic acid but putrescine.

Those cells that were capable of accumulating both catecholamines and acetylcholine were recloned under various conditions. Three classes of clones were observed: (1) Those that retained newly synthesized acetylcholine. (2) Those that retained both, newly synthesized catecholamines and newly synthesized acetylcholine. (3) Those that did not accumulate either of them. Not a single clone with the ability to accumulate only newly synthesized catecholamines was isolated. The cell hybrids were also tested for their choline acetyltransferase (CAT) and tyrosine hydroxylase activities (TH). Lines able

to accumulate catecholamines had high TH activity (70-300 pmole/mg protein/min). Among the lines accumulating acetylcholine only N18RE101 had significant CAT activity (30 pmole/mg protein/min). The other lines had low activity indicating that these lines are able to retain well, slowly synthesized acetylcholine.

The catecholamines were identified by three different chromatographic systems. In N18RE103 dopamine (DA) was found to be the major product. Small quantities of NE were also found. DOPA was not accumulated and apparently was converted immediately to DA. In N18RE1200 the only product was DA. In N18ME1 and N18ME3 DA was the major product, small quantities of NE were also found and DOPA was also accumulated to some extent.

Significance to Biomedical Research: Knowledge of the biochemistry of neuroblastoma and cell hybrids in culture provide us with understanding of neuronal mechanism and may explain certain disorders in neuronal communication and synaptic function in vivo.

Honors and Awards: None

Publications: None

Project No. Z01 HL 00006-02 LBG
1. Biochemical Genetics
2. Molecular Biology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Ultrastructure of Neuroblastoma Somatic Cell Hybrids

Previous Serial Number: NHLI-308

Principal Investigator: Mathew Daniels, Ph.D.

Other Investigators: John Minna, M.D., Mr. Doyle Mullinax (M. A.), Zvi Vogel, Ph.D., Marshall Nirenberg, Ph.D.

Cooperating Units: Microbiological Associates

Project Description:

Objectives: Previous and ongoing work in this laboratory has shown that some somatic cell hybrids between neuroblastoma and other cell types can express neuronal characteristics to varying degrees, in some cases to a greater degree than the parent cells. The objective of the present project was to extend these observations to the ultrastructural level by means of electron microscopy of the intact cell cultures of hybrid lines derived by crosses between neuroblastoma and glioma, L-cells, human fibroblasts, or embryonic nerve cells.

Methods Employed: We are applying standard transmission electron microscopic techniques to monolayer and rotation-mediated aggregate cell cultures fixed and embedded without any dislocation.

Major Findings: We have studied the ultrastructure of aggregates of several lines of neuroblastoma x Chinese hamster retina (NCE) and neuroblastoma x rat retina (NRE) somatic cell hybrids. In the NCE hybrid lines there was a wide variation in the ability to aggregate. This ability was generally correlated with the frequency of specialized intracellular junctions. Further, there appeared to be at least 2 classes of junction-forming cells, those with only small, "macula adherens" (MA) type junctions and those with both MA junctions and larger (with a narrower gap), "zonula adherens" (ZA) type junctions. These specialized junctions were also observed in aggregates of the NRE hybrids. In addition, two of these hybrid lines showed extensive neurite formation in aggregates, a feature not observed in the NCE lines.

Significance to Biomedical Research: This investigation may yield information as to the pattern of inheritance of neuronal characteristics in the somatic cell hybrids as well as the appropriateness of these cells for use as

neuronal models. This type of information is ultimately important in the attempt of this laboratory to understand the biochemical and genetic basis for nervous system function and development.

Proposed Course: We plan to describe the intercellular junctions in more detail and compare them to those of retinal and neuroblastoma cells. In addition, it should be of interest to co-aggregate with retinal cells the NRE lines which form neurites, since these cells may have more tendency for interaction.

Honors and Awards: None

Publications:

1. Daniels, M. P. and Hamprecht, B.: The ultrastructure of neuroblastoma glioma somatic cell hybrids. Expression of neuronal characteristics stimulated by dibutyryl adenosine 3',5' cyclic monophosphate. J. Cell Biol., 63, 691, 1974.

Project No. Z01 HL 00007-01 LBG
1. Biochemical Genetics
2. Molecular Biology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Ornithine Decarboxylase Induction in Neural Cells

Previous Serial Number: None

Principal Investigator: Uriel Bachrach, Ph. D.

Other Investigators: None

Cooperating Units: None

Project Description:

Major Findings: The naturally occurring polyamine, spermine, spermidine and putrescine are wide-spread in biological material, including the nervous system. Cellular polyamine levels fluctuate during the growth cycle and correlate well with cellular RNA concentrations. The rate-limiting step in polyamine synthesis is ornithine decarboxylase, which catalyzes the conversion of ornithine to putrescine. We were able to show that ornithine decarboxylase activity of neuroblastoma N115 and Glioma C6-Bu-1 cells was high in proliferating cells and declined when they reached confluency. When confluent cells were fed with fresh medium, ornithine decarboxylase activity (ODC) increased precipitiously after a lag of 2 hours and was 1000-fold higher than the basal activity, 4 hours later these changes in ODC were accompanied by changes in cellular putrescine levels. This unique increase in ornithine decarboxylase activity was accompanied by accumulation of polyamines, and by resumption of RNA synthesis. The induction of ODC, by fresh medium, could be prevented by Actinomycin D and by cycloheximide and was also accomplished by the addition of dibutyryl cAMP, isoproterenol, norepinephrine or $PGE_1$ (prostaglandin) to confluent neuroblastoma and glioma cells, respectively. Phosphodiesterase inhibitors, such as, theophylline, Ro 20-1274 [4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone] and IBMX (3-isobutyl-1-methylxanthine), also caused the induction of ODC when added to confluent cells. Since all these agents are known to bring about the accumulation of cAMP, it has been suggested that ODC induction is mediated by cAMP, which probably operates on the level of gene transcription.

Significance to Biomedical Research: The activity of ornithine decarboxylase (ODC), the initial enzyme in polyamine biosynthetic pathway, fluctuates during the growth cycle of neuroblastoma and glioma cells. The activity of the enzyme is 1000 times higher in proliferating cells compared with stationary ones. This study indicates that cAMP mediates the induction of ODC. Growth of neural cells may thus be regulated by cAMP by modulating cellular ODC and polyamine levels.

Project No. Z01 HL-00008-01 LBG
1. Biochemical Genetics
2. Molecular Biology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Protein Phosphorylation in Neuroblastoma Cells

Previous Serial Number: None

Principal Investigators: Steven Sabol, M.D., Ph.D. and Marshall Nirenberg, Ph. D.

Other Investigators: None

Cooperating Units: None

Project Description:

Major Findings: C-1300 neuroblastoma cells have been shown to exhibit a variety of electrophysiological responses to putative neurotransmitters. Clone N115 cells respond to iontophoretic application of cholinergic agents by transient hyperpolarization of the plasma membrane. Dr. H. Matsuzawa demonstrated that cholinergic agents also cause a large transient increase in the content of guanosine 3'5' monophosphate (cyclic GMP) of these cells. Both responses follow similar time courses (5-60 seconds) and are mediated by muscarinic cholinergic receptors. Because of the existence in eukaryotic cells of protein kinases activated by cyclic nucleotides, it was hypothesized that membrane potential changes in nerve or neuroblastom cells exposed to muscarinic cholinergic agents may be the result of cyclic GMP-dependent phosphorylation of membrane proteins concerned with ion transport. Preliminary studies demonstrated the existence in N115 cells of histone kinase activity which was stimulated by physiological concentrations of cyclic GMP and which could be chromatographically resolved from some of the adenosine 3'5' monophosphate (cyclic AMP) - dependent protein kinase activity. In a search for endogenous substrates for protein kinases in N115 cells, gel electrophoresis and autoradiography were employed to identify several soluble proteins which were phosphorylated in a manner dependent on cyclic AMP and to equal or lesser extent on cyclic GMP. No strictly cyclic GMP-dependent kinase substrates were found. Electrophoretic analysis of proteins from whole cells treated for various times with carbamylcholine revealed no obvious changes in phosphoproteins which could be ascribed to the elevation of cyclic GMP concentration. However, more highly resolving electrophoretic methods are currently being applied to this problem.

Significance to Biomedical Research: Through this work, an attempt is made to understand the mechanism of generation of slow postsynaptic potentials which occur in some neurons in response to muscarinic cholinergic stimulation. Such potentials are thought to be important in the control of nerve excitation.

Project No. Z01 HL-00009-01 LBG
1. Biochemical Genetics
2. Molecular Biology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Gene Expression for Neural Properties

Previous Serial Number: None

Principal Investigators: Eliahu Heldman, Ph. D. and Marshall Nirenberg, Ph.D.

Other Investigators: John Minna, M.D. and Hayden Coon, M.D. (NCI).

Cooperating Units: National Cancer Institute, LCB

Project Description:

Major Findings: During the past few years, numerous neuroblastoma cell lines were obtained and characterized with respect to neuronal properties such as transmitter synthesis, storage, catabolism, receptors and effects of receptor activation.

Additional cell lines with new neural phenotypes were generated and questions of dominance of gene expression and complementation were explored by fusing neuroblastoma with other cells and obtaining hybrid cell lines. The expression of genes for neural properties was found to be dominant with most matings. Some hybrid clones expressed the neural phenotypes of the neuroblastoma parent 50 or more cell generations after fusion; many others had specific defects in transmitter synthesis, storage, and catabolism; response to neurotransmitters, action potential reactions; and so forth. Another class of hybrids had acquired new neural properties which were not detected with parental neuroblastoma cells. For example, we previously showed that fusion of mouse neuroblastoma cells with rat glioma cells, both lacking choline acetyltransferase activity which store acetylcholine and have clear vesicles identical in appearance to those found at synaptic junctions. Additional studies now show that fusion of mouse neuroblastoma cells which lack tyrosine hydroxylase activity with cells from normal sympathetic ganglia from mouse embryos yields hybrid cells with high tyrosine hydroxylase activity that synthesize dopamine, possess muscarinic excitatory acetylcholine receptors, and have both small and large dense-core vesicles. Fusion of neuroblastoma cells with cells from normal retina yielded some cell lines that synthesize catecholamines and another that synthesizes acetylcholine. These results show that fusion of neuroblastoma cells with cells from the normal nervous system generates hybrid cells with new neural properties which have not been detected with the parental neuroblastoma cells. The new neural phenotypes

are inherited and thus far have been perpetuated in a fairly stable fashion for more than 100 cell generations. This approach would appear to be a general one that can be used to obtain cell lines with other differentiated properties that can be used to elucidate reactions that are required for cell communication.

Publications:

1. Breakfield, X. O. and Nirenberg, M. W.: Selection for neuroblastoma cells that synthesize certain transmitters. Proc. Nat. Acad. Sci., USA, 71: 2530-2533, 1974.

2. Giller, E. L., Breakfield, X. O., Christian, C. N., Neale, E. A. and Nelson, P. G.: Expression of neuronal characteristics in culture: some pros and cons of primary cultures and continuous cell lines. In: Santini, M. (Ed.) Golgi Centennial Symposium: Perspectives in Neurobiology. New York, Raven Press, pp. 603-623, 1975.

3. Breakfield, X. O.: Reserpine sensitivity of catecholamine metabolism in murine neuroblastoma clone NIE-115+. J. Neurochem., 1974, In press.

Project No. Z01 HL 00010-01 LBG
1. Biochemical Genetics
2. Molecular Biology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Naturally Occurring Anti-Tumor Antibodies

Previous Serial Number: None

Principal Investigator: Sue Ellen Martin

Other Investigators: William J. Martin, M.D., (NCI)

Cooperating Units: National Cancer Institute, VLL

Project Description:

Objectives: Detection and characterization of tumor related cell surface antigens.

Methods Employed: In vitro cultivation of tumor cells of both mesodermal and non-mesodermal origin. Antigenic analysis of the tumor cell lines using both immume and naturally occurring antibodies in the complement dependent, antibody-mediated cytotoxicity assay.

Major Findings: Normal mouse sera were shown to contain a wide variety of naturally occurring antibodies (NOA) reactive with both recently derived and long term transplantable tumor cell lines. The occurrence of NOA in sera of congenitally athymic (nude) mice indicated that the production of these antibodies was thymus independent. Absorption studies revealed that the various tumor cell lines expressed both individually distinct and shared antigens. An antigen recognized by NOA on a murine cell line derived from a neuroblastoma adrenal metastasis of a spontaneous murine ovarian teratoma was found to be present on normal brain tissue of species as diverse as man and chicken. Many of the tumor antigens recognized by NOA could not, however, be detected on normal tissues and appeared to be tumor specific. A strain of mouse was identified which had a genetically determined defect in the production of tumor reactive NOA. These mice did not have an unusually high incidence of spontaneous tumors.

Significance to Biomedical Research: While it has generally been assumed that the immume system plays an important role in the detection and elimination of nascent tumors, little evidence in support of this hypothesis has been forthcoming. The recent demonstration that nude (congenitally athymic) mice do not have an increased incidence of spontaneous tumors has seriously

challenged the concept that T cell immunity plays a crucial role in immune surveillance against tumors.

The demonstration that some NOA are directed against normal tissue antigens may indicate that naturally occurring antibodies serve some function other than immune surveillance. Such anti-self antibodies may play an important role in the prevention of tissue destructive autoimmunity.

Naturally occurring antibodies provide a very sensitive method with which to detect both tumor-associated and normal tissue antigens and a powerful tool for the identification and isolation of these components.

Proposed Course: Naturally occurring antibodies will be used for the identification and isolation of cell surface components.

Publications:

1. Martin, S. E. and Martin, W. J.: Anti-tumor antibodies in normal mouse sera. Int. J. Cancer, In press.

2. Martin, S. E. and Martin, W. J.: Interspecies brain antigen detected by naturally occurring mouse anti-brain autoantibody. Proc. Nat. Acad. Sci., USA, In press.

Project No. Z01 HL 00011-01 LBG
1. Biochemical Genetics
2. Molecular Biology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Glutamic Acid Decarboxylase in Developing Chick Retina.

Previous Serial Number: None

Principal Investigator: Fernando DeMello, M. D.

Other Investigators: Marshall Nirenberg, Ph. D.

Cooperating Units: None

Project Description:

Major Findings: The objective is to follow the appearance of glutamic acid decarboxylase activity during the course of retina differentiation and attempt to correlate it with synaptogenesis. Glutamic acid decarboxylase (GAD) activity increases and shows a remarkable elevation which levels off after hatching. The level of gamma amino butyric acid in the retina increases with the increase of GAD activity.

Significance to Biomedical Research: The role neurotransmitters play in synaptogenesis is of clear importance in any attempt to understand the function and development of the nervous system.

Proposed Course: We are now attempting to study the regulation processes involved in the development of glutamic acid decarboxylase in chick retina. Emphasis is to be made on the role of cyclic nucleotides in this process.

Publications: None

Project No. Z01 HL 00012-01 LBG
1. Biochemical Genetics
2. Molecular Biology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Muscarinic Acetylcholine Receptors in Cultured Cell Lines

Previous Serial Number: None

Principal Investigators: Marshall Nirenberg, Ph.D. and William Klein, Ph.D.

Other Investigators: None

Cooperating Units: None

Project Description:

Previous experiments have indicated that a number of cell lines isolated in the laboratory possess active acetylcholine receptors. (Unpublished results, Nirenberg, Matsuzawa, Sharma, Catterall, and Gibbons). As clonal lines present a number of advantages for studying neural phenomena, the presence of such receptors on these lines may be particularly useful in studying receptor action. The cells afford an opportunity to study the molecular basis of receptor function as well as such important aspects as short-term and long-term regulation of receptor activity. Investigating the nature of receptor function and regulation is the long-range focus for this line of experimentation. Initial results are described below.

Direct binding studies have been employed to confirm and quantitate the occurence of acetylcholine receptors in several cell lines. Radioactive quinuclidinyl benzilate (QNB), a highly sensitive and specific muscarinic antagonist, was prepared and purified and used to assay receptor concentrations in intact cells under physiological conditions. Cell lines with hyperpolarizing responses to acetylcholine and others with depolarizing responses have been measured to have 50-100 fmoles of muscarinic receptor per mg protein. Control cells with no measurable levels of receptor have been found. The assay is sensitive to less than 5 fmoles per mg protein. Evidence that QNB binds to muscarinic receptors as expected is seen in the 100-fold greater sensitivity of binding to oxotremorine than to tubocurarine. Oxotremorine and tubocurarine are drugs relatively specific for muscarinic receptors and nicotinic receptors, respectively. The dissociation constant for ($^3$H)-QNB is approximately $10^{-9}$ M. Competition experiments done with labeled atropine and various muscarinic agents are in agreement with results obtained using QNB.

Significance to Biomedical Research: A variability in receptor levels seen from day to day suggests that the amount of receptor per cell may be regulated. Preliminary experiments suggest that receptor concentration may increase with the age of culture.

Culturing cells in the presence of muscarinic agonists appears to lower the level of specific QNB binding. Also, a shorter pulse of agonist appears to desensitize the receptor, lowering QNB binding for a period lasting several hours. Removal of oxygen for 30 minutes lowers receptor levels about 50% and starvation of cultures lowers receptor levels considerably more.

Removal of $Na^{+}$ and $Ca^{+2}$ from the assay medium has no effect on oxotremorine-sensitive QNB binding. However, a large oxotremorine-insensitive component seen in complete medium is greatly reduced. This oxotremorine-insensitive binding is also lost after cell homogenization, but specific binding is also lower than can be accounted for by randomization of membrane sidedness.

Honors and Awards: None

Publications: None

Project No. Z01 HL 00013-01 LBG
1. Biochemical Genetics
2. Molecular Biology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Developmental Regulation of Excitability

Previous Serial Number: None

Principal Investigator: Jonas B. Galper, M.D. and William A. Catterall, Ph.D.

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: The objectives of this project are (1) to devise biochemically manipulable cell culture systems for studying the development of action potential generation and receptor function in excitable cells, (2) to use these cell culture systems to document the changes in action potential generation and receptor function occurring during development, and (3) to understand the role of hormonal stimuli, cell-cell interaction, and synapse formation in these developmental changes.

Methods Employed: Cultures of clonal lines of mouse neuroblastoma and rat skeletal muscle and cultures of primary embryonic chick muscle were prepared by standard procedures. Primary cultures of chick embryonic heart cells were prepared either as monolayers of individual beating cells or as suspension cultures of aggregates of a few hundred synchronously beating cells. The ion transport activity of action potential and receptor ionophores was studied using measurements of $^{22}Na^+$ influx as described previously (Project NHLI-307).

Major Findings: (1) Neuroblastoma cells logarithmically growing in either monolayer or suspension culture exhibit significant levels of action potential $Na^+$ ionophore activity indicating that this differentiated property is expressed in the dividing cell. Inhibition of cell growth with either butyric acid or dibutyryl cyclic AMP causes a marked increase in ionophore activity. (2) Primary cultures of embryonic chick skeletal muscle have undetectable levels of action potential $Na^+$ ionophore activity until at least one day after fusion of the cells into multinucleate myotubes. The observed activity is always sensitive to tetrodotoxin ($K_I$ = 1-3 x $10^{-8}$ M). Cultures of clonal lines of rat skeletal muscle have significant levels of action potential $Na^+$ ionophore activity before fusion and exhibit only a 2-3 fold increase in activity concomitant with fusion. The observed activity is always relatively

insensitive to tetrodotoxin ($K_I$ = 1-3 x $10^{-6}$ M). Thus the developmental regulation of the action potential $Na^+$ ionophore in avian and mammalian muscle appears different. (3) At least three distinct ionophores are involved in the action potential in normal adult heart: a nerve-like action potential $Na^+$ ionophore, an action potential $Ca^{++}$ ionophore which also transports $Na^+$, and a $K^+$ ionophore required for repolarization. During development *in ovo*, beating of embryonic chick hearts becomes increasingly more sensitive to inhibition by tetrodotoxin, an inhibitor of the nerve-like action potential $Na^+$ ionophore. At the same time, beating becomes less sensitive to inhibition by compound D-600 which is thought to be an inhibitor of the action potential $Ca^{++}/Na^+$ ionophore. Beating of embryonic heart cells in monolayer culture is insensitive to tetrodotoxin regardless of the age of the embryo from which the cells were obtained but the sensitivity of monolayer cells to D-600 decreases with embryonic age as *in ovo*. Despite the tetrodotoxin insensitivity of beating of monolayer heart cells, these cells have significant levels of neurotoxin stimulated $^{22}Na^+$ uptake indicating significant levels of action potential $Na^+$ ionophore activity and this $Na^+$ transport activity has normal sensitivity to tetrodotoxin ($K_I$ = $10^{-8}$ M). Thus these cells have substantial tetrodotoxin-sensitive action potential $Na^+$ ionophore activity which is not required for beating. This transport activity is also inhibited by compound D-600. The inhibition is competitive with respect to the activating neurotoxins. The $K_I$ for inhibition of uptake by D-600 increases from 5 x $10^{-8}$ M to 1 x $10^{-6}$ M with increasing embryonic age as does the $K_I$ for inhibition of beating. Thus D-600 can inhibit both action potential $Na^+$ ionophore activity and beating which is dependent on $Ca^{++}/Na^+$ ionophore activity by a common mechanism whose sensitivity changes with embryonic age. (4) Aggregate cultures of embryonic chick heart cells beat synchronously in culture. The beating becomes increasingly sensitive to tetrodotoxin with increasing age of the embryo from which the cells were derived. Aggregates whose beating is inhibited by tetrodotoxin reactivate over a period of 2 to 3 hours in culture in a process that appears to require protein synthesis. Reactivation occurs only in cultures prepared from "transitional" hearts, those whose beating is partially sensitive to tetrodotoxin. It is highly dependent on the choice of medium and serum. Reactivation in the presence of tetrodotoxin may represent a rapid response of the cells to inhibition of rhythmic activity which involves modification of components of tetrodotoxin-sensitive action potential ionophores. (5) A fraction of the aggregates of chick embryonic heart cells formed by our methods exhibit asynchronous beating superficially resembling some clinically described arrhythmias. The fraction of aggregates exhibiting arrhythmias varies with culture conditions and age *in ovo*.

Significance to Biomedical Research: The results provide new insight into the developmental regulation of membrane macromolecules involved in information transfer and processing in the nervous system and in maintenance of normal beating in heart.

Proposed Course: Planned investigations include (1) studying further the influence of cell surface interactions and cell growth rate on action potential $Na^+$ ionophore activity of neuroblastoma cells; (2) extending the study of developmental regulation in muscle to include other mammalian systems and assessing the influence of nerve on the development of muscle action potential

$Na^+$ ionophore; (3) studying in more detail the differences between the action potential $Na^+$ ionophore in heart cells that require its activity for beating and those that do not; (4) documenting the changes in activity of the action potential $Na^+$ and $Ca^{++}/Na^+$ ionophores during reactivation of heart cell aggregates made quiescent by tetrodotoxin; (5) studying the effects of nerve on the action potential ionophore activity of heart cell aggregates; and (6) developing rational procedures for inducing arrhythmias in heart cell aggregates and for pharmacologic treatment of such arrhythmias.

Honors and Awards: None

Publications: None

Project No. Z01 HL 00076-05 LBG
1. Biochemical Genetics
2. Somatic Cell Genetics
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Genetics of Cyclic-AMP Metabolism

Previous Serial Number: NHLI 308

Principal Investigator: John D. Minna, M.D.

Other Investigators: Stuart Brown, Ph. D., Thomas Marshall, Ph. D., Richard Lemons, Ph. D. and Alfred Gilman, M.D., Ph.D. (UVA).

Cooperating Unit: University of Virginia, Department of Pharmacology.

Project Description:

Major Findings: Clonal mammalian cells replicating in vitro have been phenotyped for enzymes and receptors relevant to cAMP metabolism. Cell fusion studies were then performed and hybrid cells characterized for their phenotype for these properties. To date prostaglandin $E_1$, β adrenergic responses, phosphodiesterase, and adenylate cyclase (basal and levels following hormone stimulation), as well as binding of labeled prostaglandins and catecholamines are being studied. We are concentrating at present on the dominantly inherited $PGE_1$ receptor-adenylate cyclase system in human mouse hybrid segregating either human or mouse chromosomes.

Significance to Biomedical Research: By phenotyping the hybrids for their $PGE_1$ responsiveness and then determining their chromosome composition, assignment of human and mouse genes for these metabolically important functions can be achieved.

Honors and Awards: None

Publications: Maguire, M.E., Sturgill, T. W., Anderson, H. J., Minna, J.D. Gilman, A. G.: Hormonal control of cyclic AMP metabolism in parental and hybrid somatic cells. Advances in Cyclic Nucleotide Research, Vol. 5, 1977.

Project No. Z01 HL 00077-03 LBG
1. Biochemical Genetics
2. Somatic Cell Genetics
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Expression of Type C virus in Human-Mouse Cell Hybrids

Previous Serial Number: NHLI-311

Principal Investigators: John D. Minna, M.D., Thomas H. Marshall, Ph.D., Richard Lemons, Ph.D., Ronald Yasbin, Ph.D., and Stuart Brown, Ph.D.

Other Investigators: S. H. Wilson, Ph.D. and A. Gazdar, M.D.

Cooperating Units: NCI

Project Description:

Human x rodent hybrid cell lines segregating either human or rodent chromosomes have been analyzed for the expression of type C particle markers (oncornavirus) using RNA dependent DNA polymerase (RDDP) assays, specific antisera against viral proteins, electron microscopy, and viral biologic activity. The hybrid cell experiments have explored three general systems: 1) the regulation of characterized rodent virus by human genes; 2) the ability of the hybrid cells to support the replication of exogenously applied virus; 3) the de novo (spontaneous or induced) production of new virus by the hybrid cells. In order to undertake these studies with clinically available material techniques were established that allow the generation of hybrid cells after fusion to small samples of normal or malignant tissue taken directly from patients. The following results have been obtained. We can reproducibly generate large numbers of mouse x human hybrids with all leukemia cell types. We have demonstrated that human genes can regulate the production of viral RDDP but have no apparent effect on expression of the main viral structural protein.

By analyzing hybrids segregating human chromosomes it is possible to assign these regulatory genes to human chromosomes 14, 21 and possibly 12. Mouse xenotropic virus able to replicate in human but not mouse cells has a complex genetic control pattern when tested in human x mouse hybrid cells. In addition, we find human genes can block the induction of type C viral genes from mouse genetic integration sites, and can alter the host range of mouse virus. Thus xeno and ecotropic control involves surface receptors, intracellular preintegration regulation, post integration control, and host range modification. By applying viruses known to cause neoplasia in primates (woolly monkey, gibbon ape lymphoma virus) to hybrid cells segregating human chromosomes we

are systematically looking for the human genes required for their replication and integration. Hamster-mouse hybrids losing mouse chromosomes are also being studied to determine which mouse genes are required for viral gene replication. Clones able and clones unable to support murine leukemia viral replication have been isolated. This should widely extend the previous mouse genetic studies.

Significance to Biomedical Research: These studies will allow a genetic analysis of human and mouse genes important in the regulation, production, replication and structure of virus particles known to play an important role in growth regulation, neoplasia and possible differentiation. In addition, they demonstrate the highly evolved mechanisms for oncogenic virus control in humans.

Publications:

1. Gazdar, A. F., Russell, E. K., and Minna, J.: Biologic properties of a type C virus isolated from a human x mouse hybrid cell line. Proc. Soc. Exp. Med. Biol., In press.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Chromosome Segregation in Hybrid Cells

Previous Serial Number: NHLI-313

Principal Investigator: John D. Minna, M.D.

Other Investigators: Thomas Marshall, Ph.D., Stuart Brown, Ph.D., Richard Lemons, Ph.D., Ronald Yasbin, Ph.D., and Hayden Coon, M.D. (NCI)

Cooperating Units: Laboratory of Cell Biology, NCI

Project Description:

We have found that chromosome segregation patterns can be varied by proper selection of the parent cells introduced into a cell fusion reaction. The fundamental principal we have discovered is that fusion of a transformed tissue culture line to fresh normal cells from a laboratory animal results in the segregation of the chromosomes of the normal cell parent. This allows gene mapping by testing of the hybrid lines as they segregate chromosomes. The phenomonon itself is of fundamental biologic importance with respect to control of foreign genetic information. The linkage studies will allow among other things the development of a genetic evolutionary map for the mammalian chromosomes. At present we have assayed 21 different isozymes in mouse x hamster, mouse x human, and chinese hamster x human hybrid cells losing various patterns of chromosomes. This has enabled us to derive new linkage data for the mouse. Of importance: 1) we have demonstrated that at least 15 different isozymes are asyntenic in the mouse; 2) that genes linked in the human that are on different chromosome arms are unlinked in the mouse; and 3) that at least some genes on the same chromosome arm in the human are linked in the mouse.

In addition, human x mouse hybrid cells have been generated by mating mouse tissue culture lines to cells taken from human patients with dominantly inherited genetic diseases and malignant leukemic cells. These are being analyzed for their pattern of chromosome segregation. To date we have found that segregation of human chromosomes in such hybrids is non-random with preferential elimination of some human chromosomes while others are selectively retained.

Significance to Biomedical Research: These observations are of fundamental biologic importance and will allow the construction of a mammalian evolutionary

chromosome map. This genetic approach to malignancy and other genetic disorders is novel and should uncover human genes important for growth and virus regulation.

Honors and Awards: None

Publications:

1. Minna, J. D. and Coon, H. G.: Human x mouse hybrid cells segregating mouse chromosomes and isozymes. Nature, 252, 401-404, 1974.

Project No. Z01 HL 00079-02 LBG
1. Biochemical Genetics
2. Somatic Cell Genetics
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Genetic Analysis of Differentiation Using Cell Hybrids

Previous Serial Number: NHLI-305

Principal Investigators: John D. Minna, M.D. and Richard Lemons, Ph.D.

Other Investigators: A. Deisseroth, M.D., A. Neinhuis, M.D., and F. Anderson, M.D.

Cooperating Units: Molecular Hematology Branch, NHLI

Project Description:

Genetic analysis of differentiated functions related to the erythropoetic system was carried out using somatic cell hybridization techniques. Friend virus transformed murine erythroleukemia cells replicate as clonal lines in vitro and express many differentiated functions of red cells. A 6-thioguanine resistant mutant, deficient in hypoxanthine phosphoribosyl transferase, was isolated and shown to be unable to replicate in selective HAT medium, produced hemoglobin after induction with dimethylsulfoxide, and exhibited high levels of acetylcholinesterase. These cells were mated to mouse and human fibroblasts not expressing these differentiated functions and the resultant hybrids isolated and characterized. The hybrids before chromosome segregation (with retention of chromosome bearing hemoglobin structural genes) were found to have extinguished these differentiated functions. The specific level of gene regulation was determined by molecular hybridization experiments. The hybrids were found to produce no hemoglobin messenger RNA.

Significance to Biomedical Research: This represents the first determination of the level of gene regulation following extinction of differentiated functions in hybrid cells.

Honors and Awards: None

Publications: None

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Genetic Analysis of Hyperlipidemias Using Cell Hybrids.

Previous Serial Number: NHLI 312

Principal Investigator: John D. Minna, M. D.

Other Investigators: Thomas Marshall, Ph. D., Stuart Brown, Ph. D., Richard Lemons, Ph. D. and Howard Sloan, M.D. (MDB)

Cooperating Units: NHLI, Molecular Diseases Branch

Project Description:

Major Findings: Fibroblasts from patients with homozygous type II hypercholesterolemia bearing the defect in regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase (EC 1.1.1.34, HmGCoA) were fused to mouse cells not demonstrating this defect and hybrid clones isolated. Hybrid lines segregating human chromosomes will be phenotyped for the defect and for their human chromosome content. We are analyzing the lines for their human gene content. To date 21 different human isozymes assigned to 15 different human chromosomes have been tested in 15 independently isolated hybrid clones.

Significance to Biomedical Research: Analysis of the chromosome and HmGCoA phenotype should allow a systematic genetic analysis of human genes involved in the production and regulation of lipoproteins and chromosome assignment of the gene for this clinically important disorder.

Honors and Awards: None

Publications: None

Project No. Z01 HL 00081-01 LBG
1. Biochemical Genetics
2. Somatic Cell Genetics
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Bacteriophage Resistance in Transformed Bacillus subtilis

Previous Serial Number: None

Principal Investigator: Ronald E. Yasbin, Ph.D.

Other Investigators: Frank E. Young, M.D., Ph.D.

Cooperating Units: University of Rochester, Department of Microbiology

Project Description:

B. subtilis W23, unlike B. subtilis 168, is resistant to bacteriophages SPP1, SP02, ϕ105 and ϕ29. This resistance has been attributed to the inability of these bacteriophages to absorb the cell wall of strain W23. On the other hand, bacteriophages ϕe, ϕ25, SP01, and SP82 plaque with an efficiency of $10^{-1}$ to $10^{-3}$ on strain W23 as compared to strain 168. However, these four bacteriophages absorb equally well to the cell walls of B. subtilis 168 and B. subtilis W23. Strain 168 was transformed, at a very low frequency ($10^{-6}$), to SPP1/SP02 resistance by DNA isolated from strain W23. Characterization of four isolated transformants revealed that all were completely resistant to bacteriophages ϕ105 and ϕ29. One of the strains (RUB824) was completely resistant to both bacteriophages SPP1 and SP02 while RUB823 was completely resistant to bacteriophage SPP1 and only partially resistant to bacteriophage SP02. In addition, the remaining two transformed strains (RUB821 and RUB822) were only partially resistant to both bacteriophages SPP1 and SP02. Strain RUB824 was completely resistant to bacteriophage SP82 while partially resistant to bacteriophage SP01. On the other hand, strains RUB821, 822, and 823 were completely resistant to bacteriophage SP01 while being only partially resistant to bacteriophage SP82. The successful absorption of bacteriophage to these transformed strains does not necessarily result in a successful infection. These results indicate the complex nature of bacteriophage resistance in B. subtilis. Additionally, utilizing the processes of transformation and transfection, it appears that bacteriophage DNA is discriminated against in these four strains. These results suggest the acquisition and/or activation of nucleases in the transformed strains which were not present in the parent 168 strain. The action of these nucleases is presently under investigation.

Significance to Biomedical Research: This research is designed to elucidate the types of molecules used as viral receptors on cell surfaces.

Publications: None

Project No. Z01 HL 00082-01 LBG
1. Biochemical Genetics
2. Somatic Cell Genetics
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Type C Virus Particles in Normal and Diseased Humans.

Previous Serial Number: None

Principal Investigators: Stuart Brown, Ph. D., Richard Lemons, Ph. D. and John D. Minna, M.D.

Other Investigators: Dr. R. Young (NCI) and Dr. P. Schein (GUH)

Cooperating Units: National Cancer Institute, Medicine Branch and Georgetown University Hospital, Department of Oncology

Project Description:

Major Findings: Type C virus particles have been implicated in neoplastic disease, growth regulation, and intracellular information transfer. Certain biochemical, biologic and immunologic characteristics of these particles allow for their identification. To be clinically useful, assay for such particles must be performed using small samples of easily obtained patient material, collected under conditions readily available, followed by a minimum of processing. We have established these conditions as well as the assay for one such marker, RNA dependent DNA polymerases in small samples of peripheral blood body fluid, or tissue and then assayed a large number of normal individuals. Of interest we find in plasma, serum and platelets a particle sedimenting at 40,000 x g but not 10,000 x g which contains a DNA polymerase activity. By a series of tests we have established that the assay for this activity is reproducible, sensitive, and can be quantitatively performed on crude biologic preparations. Partial characterization of the activity shows it to be similar to a class of cytoplasmic DNA polymerases (type III, or gamma), that are related but not identical to type C virus DNA polymerase. The source of the particle is at present unknown but could be released from platelets during hemostasis. Its biologic role is unknown but, because of its transmission during transfusion therapy, and because of its ability to circulate around the body, could play an important physiologic as well as pathologic role. A large number of samples have been collected from patients with neoplastic and non-neoplastic diseases of possible type C viral origin (eg. systemic lupus erythematosis) and are being assayed using the above described techniques. By fusing human cells to mouse cells carrying an integrated sarcoma virus genome, a hybrid cell can be made that will be of potential use for biologic detection of virus particles. When the hybrid cell is infected with a replicating type C virus (leukemia virus) the sarcoma

virus can be "rescued" and transforms the cells' morphology yielding a scorable "focus or plaque". Because of the human genes present human derived virus may be introduced into such a hybrid, and this cell can be used for focus formation assay.

Honors and Awards: None

Publications: None

Project No. Z01 HL 00151-05 LBG
1. Biochemical Genetics
2. Macromolecules
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Biology of Cyclic Nucleotides in E. coli

Previous Serial Number: NHLI-309

Principal Investigator: Alan Peterkofsky, Ph.D., James Harwood, Ph.D., and Jose Gonzales, Ph.D.

Other Investigators: Mrs. Celia Gazdar

Cooperating Units: None

Project Description:

Major Findings: Cyclic AMP (cAMP) plays a key role in metabolic control in E. coli. It is required for transcription of the genes for many induced enzymes. Glucose is effective in regulating cAMP levels. Previous studies showed an inverse correlation between the presence of glucose in culture medium and the accumulation of cAMP. This observation could not be explained by the action of glucose as a repressor of adenylate cyclase synthesis, as a stabilizer of cAMP phosphodiesterase, or as a direct inhibitor of adenylate cyclase activity in cell-free preparations. Our recent development of an in vivo assay for adenylate cyclase provided a basis for further exploring the inhibitory action of glucose in intact cells. With this assay it was possible to show that, while glucose does not affect adenylate cyclase in vitro, it rapidly inhibits the enzyme activity in intact cells. Extensive metabolism of glucose is not required since α-methyl glucoside also inhibits adenylate cyclase in vivo. Dose-response studies indicate that low concentrations of glucose lead to essentially complete inhibition of adenylate cyclase activity while only moderately decreasing intracellular cAMP levels. We therefore concluded that the decreased cellular cAMP levels resulting from glucose addition to intact cells can be accounted for by inhibition of adenylate cyclase without any significant effect on cAMP phosphodiesterase or the transport of cAMP from the cells into the medium.

When E. coli B is grown in glucose-supplemented medium, it possesses adenylate cyclase activity which can be inhibited by glucose but relatively few other compounds. When the bacteria are grown on a variety of other compounds such as fructose, mannitol, or lactose, the organisms contain adenylate cyclase activity which is inhibited by that carbon source, while maintaining the capacity of the enzyme to be inhibited by glucose. Those compounds which are effective as inhibitors of adenylate cyclase in bacteria

grown under various conditions are also effective in controlling cellular cAMP levels. A comparison of the kinetics of induction of the transport system for mannitol and the acquisition of mannitol-sensitivity of adenylate cyclase suggested a relationship of the two processes. Other studies indicated that utilization of substrate through a transport system is required for adenylate cyclase inhibition.

Significance to Biomedical Research: We have established that adenylate cyclase in broken cell preparations shows no regulation by effectors; however, in intact cells, glucose effectively inhibits adenylate cyclase. Sugars other than glucose will inhibit adenylate cyclase provided their transport systems are present. Our currently available data suggest that the membrane-bound adenylate cyclase of E. coli represents a powerful model system for the regulation by effectors of adenylate cyclase. Further studies in this system may provide insight into the mechanism by which receptor-bound hormones influence adenylate cyclase.

Publications:

1. Peterkofsky, A. and Gazdar, C.: Glucose inhibition of adenylate cyclase in intact cells of Escherichia coli B. Proc. Nat. Acad. Sci., USA, 71, 2324-2328, 1974.

2. Peterkofsky, A., Harwood, J., and Gazdar, C.: Inducibility of sugar sensitivity of adenylate cyclase of E. coli B. J. Cyclic Nucleotide Res., 1, 11-20, 1975.

Project No. Z01 HL 00152-02 LBG
1. Biochemical Genetics
2. Macromolecules
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Mechanism in Protein Synthesis

Previous Serial Number: NHLI-310

Principal Investigators: Chandan Prasad, Ph. D. and Alan Peterkofsky, Ph. D.

Other Investigators: None

Cooperating Units: None

Project Description:

Major Findings: Pyroglutamic acid (pGlu) occurs at the amino-terminus of peptides and proteins including immunoglobulin chains. While studies on the initiation of protein synthesis in eucaryotic cells have shown that methionine is the initiating amino acid, the initiation of synthesis of proteins with blocked N-terminal amino acids was not well understood. We have now been able to show that methionine transiently labels the amino terminus of an immunoglobulin light chain which contains amino-terminal pGlu. We have therefore concluded that methionine is the initiator amino acid for the synthesis of mouse plasmacytoma light chain containing N-terminal pGlu.

Significance to Biomedical Research: Methionine is the initiator amino acid for the synthesis of an immunoglobulin light chain which contains amino-terminal pyroglutamic acid.

Proposed Course: We will continue to explore the mechanism of synthesis of pyroglutamic acid in proteins and peptides.

Publications:

1. Prasad, C. and Peterkofsky, A.: Initiation by methionine of mouse immunoglobulin light chain containing $NH_2$-terminal pyroglutamic acid. J. Biol. Chem. 250: 171-174, 1975.

ANNUAL REPORT OF THE
LABORATORY OF BIOCHEMISTRY
NATIONAL HEART AND LUNG INSTITUTE
July 1, 1974 through June 30, 1975

## SECTION ON ENZYMES

Research in the Section on Enzymes is concerned with studies on biochemical mechanisms of cellular regulation and mechanisms of enzyme action.

### Biochemical Mechanisms of Cellular Regulation.

(A) Glutamine Synthetase. Regulation of glutamine synthetase in E. coli is mediated by a "closed" bicyclic nucleotidylylation cascade. One cycle involves the uridylylation and deuridylylation of the regulatory protein, $P_{II}$, which is catalyzed by uridylyltransferase (UT) and uridylyl removing (UR) enzyme respectively. The other cycle involves adenylylation and deadenylylation of glutamine synthetase (GS) both of which are catalyzed by adenylyltransferase (ATase). Coupling of the two cycles derives from the fact that the unmodified form of $P_{II}$ ($P_{IIA}$) stimulates adenylylation activity of ATase, whereas the uridylylated $P_{II}$ ($P_{IID}$) stimulates the deadenylylation activity of ATase. A steady state analysis of this unique bicyclic cascade showed that for any given metabolic state the level of glutamine synthetase adenylylation is determined by the relationship [Eq (1)],

(1) $GS_{\bar{n}} = \dfrac{12\ k_1 k_3 U_R}{k_2 k_4 U_T + k_1 k_3 U_R}$, in which $\bar{n}$ = the average number of covalently bound adenylyl groups per mole of GS; $k_1$, $k_2$, $k_3$, and $k_4$ are the specific rate constants for the deuridylylation, uridylylation, adenylylation and deadenylylation reactions respective, and $U_T$ and $U_R$ denote the uridylyltransferase and uridylyl removing enzyme activities, respectively. Bearing in mind that the activity of GS is inversely proportional to the state of adenylylation, this equation illustrates the enormous allosteric control potential of this cascade; variations in any one or all of the 6 parameters in response of fluctuations in the concentrations of multiple allosteric effectors will lead to different state levels of adenylylation.

With the separation and purification of GS, ATase, $P_{II}$, and the UTase·UR enzyme complex it has become possible to examine the effects of various metabolites on each step in the cascade.

The $P_{IIA}$ supported adenylylation of GS by ATase ($k_3$ in equation (1)) is inhibited synergistically by $P_{IID}$ and α-ketoglutarate, and is stimulated by glutamine, methionine and tryptophan. The $P_{IID}$ stimulated deadenylylation ($k_4$ of equation (1)) requires the presence of α-ketoglutarate and ATP, and is inhibited by glutamine, UTP, P-enolpyruvate, and 3-phosphoglycerate. The uridylylation of $P_{II}$ ($k_2$ of equation (1)) is stimulated by α-ketoglutarate, ATP, and is inhibited by glutamine and orthophosphate; whereas the deuridylylation of $P_{IID}$ ($k_1$ of equation 1) is stimulated by $Mn^{2+}$ and glutamine and is inhibited by CMP, UMP, and CoA. A high molecular weight endogeneous inhibitor of UR activity was partially purified and characterized as a relatively

specific CMP and UMP binding compound. Activity of this binding substance is resistant to treatment with proteases, nucleases, phospholipases, and lysozyme. The demonstration that there are reciprocal effects of glutamine and α-ketoglutarate at almost every step in the cascade, emphasizes the key role of these substances in regulating GS activity.

Studies on the mechanism of cumulative feedback inhibition of unadenylylated glutamine synthetase were continued. From detailed kinetic analyses, inhibition constants were obtained for ATP, CTP, AMP, histidine, tryptophan, and alanine. Determinations of the coefficients, $\alpha$, which reflects the degree of interaction between two inhibitors that bind to separate sites on the enzyme, disclosed that ATP and CTP react at a common site whereas AMP, histidine, and tryptophan each react at different sites on the enzyme. Furthermore, it was shown that at infinite concentrations, AMP, alanine, and glycine produce complete inhibition, but inhibition by histidine is only 50%. In general, the results support earlier conclusions that there are separate binding sites on the enzyme for most inhibitors, but in addition they show that there is considerable interaction between most binding sites.

Earlier work from this laboratory determined that the genes designating glutamine synthetase (GS) glutamate synthase (GAT), and glutamate dehydrogenase (GDH) do not constitute a contiguous operon in E. coli and may be located at approximate map positions 77', 50', and 21' respectively. Enzymic analysis of a group of revertants from gln- to $gln^+$ revealed a thermolabile GS in each case thereby substantiating that the gene locus at 77', gln A, is the structural gene for GS. Two of the revertants with thermolabile GS exhibited poor derepression on limiting $NH_3$ although the adenylylation values were low as expected. To reconcile these data with the autogenous regulation scheme proposed by Magasanik et al. the scheme should be expanded to include a positive activation of GS as well as repression by adenylylated GS. Results of preliminary investigations or the relationship between methionine sulfoxamide resistance and GS derepression suggest that if the structural gene for GS is autogenously regulated, it functions in cooperation with some other element - perhaps a component of GAT.

Significance of Cascade Control Processes.

The glutamine synthetase bicyclic cascade system is unique since the two interconvertible forms of $F_{II}$ are oppositional in their capacities to stimulate ATase to catalyze adenylylation and deadenylylation of GS. More conventional cascade systems are of the type involved in the activation of muscle phosphorylase. Here regulation is mediated by cyclic covalent modification reactions in which only the active form of an enzyme in one cycle is a catalyst for the covalent modification of an enzyme in the next. Steady state analysis of such systems shows that when the cascade involves (n-1) successive cyclic covalent modification reactions and an allosteric activation of the first enzyme in the series, then under steady state equilibrium conditions, the fraction of the modified form of the target enzyme ($E_{na}$) is described by Eq. (2),

$$\frac{E_{na}}{E_n} = \frac{1}{\left(\frac{K_d}{e}\right) + 1\left(\frac{k'_r}{k'_f}\right)^{n-1} \left(\frac{k'_r}{k'_f}\right)^{n-2} + \cdots \frac{k'_r}{k'_f} + 1} \qquad (2)$$

with the assumptions that: (a) the catalytic constants for the forward step, $k'_f = k_{1f} E_1 = k_{2f} E_2 = \cdots k_{(n-1)f} E_{(n-1)}$, where $k_{1f}$, $k_{2f}$, $\cdots$ $k_{(n-1)}$ are specific rate constants for successive forward steps in the cycles and $E_1$, $E_2$, $\cdots$ $E_n$ are total concentrations of enzymes undergoing activation at the first step, first cycle and (n-1) cycle respectively; (b) the catalytic constants for the regeneration of the unmodified forms, $k'_r = k_{1r} R_1 = k_{2r} R_2 = \cdots k_{(n-1)r} R_{n-1}$, where $k_{1r}$, $k_{2r}$, $\cdots$ $k_{(n-1)r}$ are specific rate constants for the successive regeneration steps and $R_1$, $R_2$, $\cdots$ $R_{(n-1)}$ are concentrations of enzymes catalyzing the regeneration steps. Equation (2) demonstrates the tremendous amplification potential of such cascade systems. It follows that the concentration of effector, e, required to produce 50% conversion of $E_n$ to $E_{na}$, decreases in such a manner that log $e_{0.5}$ is inversely proportional to the number of cycles in the cascade. Thus, when $K_d$ = 1 mM and $k'_r/k'_f$ = 0.1, $e_{0.5}$ is approximately equal to 1, 0.1, 0.01, and 0.001 mM for a 0, 1, 2, and 3 cycle cascade, respectively. In addition to this amplification capacity, such systems exhibit an enormous capacity for allosteric control. It is evident from Eq. (2) that positive or negative allosteric interactions with any one or all of the several enzymes in the cascade will affect the catalytic constants of these enzymes and thereby regulate the over-all ratio of $k'_r/k'_f$, which in turn determines the degree of amplication.

(B) Regulation of Enzyme Levels. Among the more important mechanisms of metabolic regulation are those concerned with the regulation of enzyme levels. The concentration of any particular enzyme in the cell reflects balance between its de novo synthesis and its degradation. In a continuing effort to develop a convenient model system for studying the regulation of specific enzyme degradation, the mechanisms that underlie the disappearance of some key enzymes in nitrogen metabolism is being investigated in resting cultures of E. coli and Klebsiella aerogenes subjected to conditions of nitrogen starvation.

a. Inactivation of aspartokinases in E. coli. Studies with suspensions of nitrogen starved, permeabilized (toluene treated) E. coli cells have shown that the selective inactivation of both the lysine-sensitive and the threonine-sensitive aspartokinase isozymes is dependent upon a carbon source and is inhibited by anaerobiosis, EDTA, HCN, and chloramphenicol. A soluble enzyme system that catalyzes inactivation of the threonine-sensitive but not the lysine-sensitive aspartokinase has been partially purified from cell free extracts. A heat stable factor that is essential for this enzyme catalyzed inactivation was isolated from boiled extracts and was identified as glycerol. The nature of the inactivation process in under investigation.

b. Inactivation of the lysine-sensitive aspartokinase and glutamine synthetase in K. aerogenes. Inactivation of the lysine-sensitive aspartokinase and glutamine synthetase activities in K. aerogenes is induced by nitrogen starvation. The loss of either enzyme activity is dependent upon the presence of an energy source, is inhibited by dinitrophenol and is greatly

stimulated by concentrations of chloroamphenicol that inhibit growth and protein synthesis. The loss in glutamine synthetase activity is associated with a loss of cross reactivity with glutamine-specific antibodies, suggesting that the loss in activity could be due to protein degradation. The results suggest further that the chloramphenicol induced enzyme degradation is due to inhibition of the de novo synthesis of these enzymes, thereby upsetting the balance between synthesis and degradation.

(C) The Mechanism of Enzyme Action.

a. Glutamine synthetase. The mechanism of glutamine synthetase catalysis has been a subject of considerable controversy. Meister has proposed that the reaction proceeds by a sequential mechanism in which ATP and glutamate react first to produce an enzyme bound glutamyl-P intermediate which then reacts with ammonia to produce glutamine and Pi, whereas Boyer concludes that the reaction occurs by a concerted mechanism in which all substrates (ATP, glutamate and ammonia) react simultaneously on the enzyme to produce a transition state intermediate which then decomposes to yield the products. With the application of fast reaction techniques and other physical and chemical methods the ability of glutamine synthesis to catalyze 5 different reactions has been studied in detail. From the experimental data all 5 reactions can be explained by an integrated mechanism in which all reactions occur at the same catalytic site. By following the changes in intrinsic fluorescence due to substrate binding and the overall biosynthetic reaction, the $k_{cat}$ and individual rate constants of the reaction catalyzed by the $Mg^{2+}$ activated enzyme could be determined. From the biphasic nature of the kinetic data obtained in the absence of ammonia it is obvious that two different intermediates are formed. However, in the presence of limiting ammonia only the fast forming intermediate is observed. From the time required for consumption of ammonia and the kinetics obtained when ammonia is added to the enzyme-Mg-ATP-glutamate complex, it is deduced that ammonia reacts only with the second intermediate. Although these and the other results could be explained by either the Meister or the Boyer hypothesis, they are more compatible with the postulate that glutamyl-P is an intermediate.

b. Role of vitamin $B_{12}$ coenzyme in conversion of $\alpha$-leucine to $\beta$-leucine. The catabolism of the branched chain amino acids, leucine, isoleucine, and valine, is incompletely understood. Certain inborn errors of metabolism have been described which implicate faulty catabolism of these amino acids; chief among these are maple syrup disease and isovaleric acidemia. In an effort to investigate the mechanism of catabolism of these amino acids strains of Clostridia have been isolated that can utilize these amino acids as a sole source of carbon, nitrogen and energy for growth. Evidence was obtained supporting the conclusion that leucine degradation in one of these organisms, Clostridium sporogenes, strain EC-9, occurs by the following mechanism: $\alpha$-leucine $\rightarrow$ $\beta$-leucine $\rightarrow$ $\beta$-ketoisocaproate $\rightarrow$ acetate + isobutyrate. The mutase catalyzing the conversion of $\alpha$-leucine to $\beta$-leucine has been partially purified and has been shown to require $B_{12}$-coenzyme for activity. This is the first time that $B_{12}$-coenzyme has been implicated in an $\alpha \rightarrow \beta$ mutase reaction. The mutase reaction has been detected in livers of rats, sheep and the Rhesus monkey, and in rat kidney. It could not be detected in either chicken or dog livers.

Project No. Z01 HL 00201-04 LB
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Metabolism of the Branched-Chain Amino Acids

Previous Serial Number: NHLI-4

Principal Investigator: J. M. Poston

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: The catabolism of the branched-chain amino acids, leucine, isoleucine, and valine, is incompletely understood. Catabolic pathways have been outlined in bovine and rat liver and one or two enzymes have been partially purified and studied. Certain inborn errors of metabolism have been described which implicate faulty catabolism of these amino acids, chief among these are maple syrup disease and isovaleric acidemia. These amino acids have been shown to participate in the Stickland reaction and many organisms are capable of fermenting these compounds in Stickland pairs. Until recently, a fermentation in which one of these amino acids serves both as the carbon and energy source had not been described. Several strains of organisms have been isolated which grow on leucine in a single amino acid fermentation. The objectives of this project are to establish the fermentation pathways of leucine and the other branched-chain amino acids in these organisms and to examine the enzymes responsible for the various metabolic steps in these fermentations.

Major Findings:

As reported previously, when cells or extracts of cells of Clostridium sporogenes strain EC-9 (one of the strains that ferment leucine) are incubated with L-leucine, several metabolic products are formed that are consistent with the pathway reported in mammals. The production of isobutyrate, however, could not be explained on the basis of the mammalian pathway, for it does not arise in any direct way from leucine. It was postulated that the pathway

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

might be operating in C. sporogenes. When evidence was sought to support the postulated mechanism, it was found, among others, that incubation of extracts with labelled leucine gave rise to labelled β-ketoisocaproate, incubation of extracts with increasing amounts of β-ketoisocaproate gave rise to increasing

amounts of acetate, and incubation of extracts with β-leucine gave rise to substrate-dependent production of α-leucine.

Direct conversion of α-leucine to β-leucine was demonstrated by the formation of labelled β-leucine when radioactive α-leucine was incubated with extracts of C. sporogenes. Because β-leucine is very difficult to measure directly, most investigations of the enzyme carrying out the interconversion of α- and β-leucine have used the reverse reaction in which β-leucine was the substrate and the α-leucine formed was measured by reaction with nihnydrin. In general, total ninhydrin reaction has been measured, but the actual formation of α-leucine has been demonstrated to parallel the total ninhydrin reaction. This was done using the automatic amino acid analyzer.

Initial experiments reported last year have been extended to explore the involvement of cobalamin with the mutase reaction. Both the forward and reverse mutase reactions are inhibited by intrinsic factor. When coenzyme-$B_{12}$ is added to the incubations there is a marked stimulation of the reverse reaction. Iron does not seem to be involved in this mutation nor is there any effect upon addition of S-adenosylmethionine. Whether pyridoxal phosphate is involved in the mutase reaction is not yet certain. This is the first known example of an α-β mutation that is $B_{12}$-dependent. Other similar mutations are stimulated by iron and pyridoxal phosphate alone, and are unaffected by the presence or absence of any $B_{12}$ coenzyme.

The leucine mutase activity can be fractionated with ammonium sulfate and recovered on gel filtration but, when it has been treated with DEAE-cellulose, the activity recovered has not been stable to freezing or storage. Activity levels in extracts prepared from various batches of cells vary widely. It is not yet clear why, but both the stage of the culture (i.e., whether or not sporulation has begun to occur) and the nutrition of the cells seem to influence the activity levels.

Other organisms have been surveyed for leucine mutase activity and Clostridium lentoputrescens was found to be a good source. Several other clostridia including C. kluyveri and a choline-fermenting clostridium had low levels of the activity. C. sticklandii and C. propionicum had no demonstrable activity.

In view of the importance of the liver in catabolizing leucine in humans, several species were examined for leucine mutase activity. Rat, sheep, and Rhesus monkey livers had the activity, but chicken and dog livers did not. Rat kidney also has appreciable activity.

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. The nature of the $B_{12}$ involvement will be established. The β-leucine deaminase will be examined and the fate of the nitrogen will be determined. The β-ketoisocaproate cleavage enzyme will be examined and its

cofactors established. The distribution of this pathway in nature will be explored and it will be determined if it plays any part in human metabolism.

Relevance to Biomedical Research:

This study impinges on at least two areas of medical concern, 1) the mode of action of vitamin $B_{12}$ in its metabolic roles, and 2) the means by which organisms catabolize food materials. This 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 case 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.

Keyword Descriptors:

Branched-chain Amino Acids, α-Leucine, β-Leucine, Clostridium sporogenes, Clostridium lentoputrescens, Cobalamin, Coenzyme-$B_{12}$, Leucine Mutase.

Honors and Awards: None

Publications:

1. J. Michael Poston and Thressa C. Stadtman: Cobamides as Cofactors: Methylcobamides and the Synthesis of Methionine, Methane, and Acetate. In Babior, Bernard M. (Ed.): Cobalamin: Biochemistry and Pathophysiology. New York, John Wiley and Sons, Inc. 1975, pp. 111-139.

Project No. Z01 HL 00202-04 LB
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Title: Kinetics and Mechanisms of Biochemical Reactions

Previous Serial Number: NHLI-10

Principal Investigators: P. B. Chock
Sue Goo Rhee

Other Investigators: None

Cooperating Units: M. Greifner, Biomedical Engineering & Instrumentation Branch, Division of Research Service, NIH
S. Chock and E. Einsenberg, Lab of Cell Biology, National Heart & Lung Institute, NIH

Project Description:

Objectives: 1) To set up a laboratory for the study of fast kinetics of reactions, particularly for studying individual steps of enzymic reactions and protein-ligand interactions. 2) With this fast kinetic technique and other physical and chemical methods, to elucidate the biochemical action of glutamine synthetase from Escherichia coli. 3) To study the kinetics and mechanism of DNA-repressor interaction utilizing the fluorescence technique.

Major Development:

1. A stopped-flow cell with a dead time of 500 μ sec have been designed and built. The short dead time is accomplished under mild conditions, such as 60 psi of driving pressure. In addition, the signal output is directly processed by a PDP 11 computer and displayed on a Tektronix 4010-1 computer display terminal. The latter set up has decreased enormously the number of man-hours required to analyze the data obtained.

2. High voltage discharge temperature-jump machine is in operation with an improved sensitivity for the optical density and fluorescent detectors.

Major Findings:

1. We have shown experimentally that an integrated mechanism can be used to explain reactions (1) to (5) catalyzed by the unadenylylated glutamine synthetase from E. coli.

$$\text{L-Glutamate} + \text{ATP} + \text{NH}_3 \overset{\text{Me}^{2+}}{\rightleftharpoons} \text{L-Glutamine} + \text{ADP} + \text{P}_i \qquad (1)$$

$$\text{L-Glutamine} + NH_2OH \underset{\text{ADP, } P_i \text{ or } As_i}{\overset{Me^{2+}}{\rightleftharpoons}} \gamma\text{-glutamylhydroxamate} + NH_3 \qquad (2)$$

$$\text{L-Glutamate} + ATP \overset{Me^{2+}}{\rightleftharpoons} \text{pyrrolidone carboxylate} + ADP + P_i \qquad (3)$$

$$\text{L-Glutamine} + H_2O \xrightarrow[\text{ADP, } As_i]{Me^{2+}} \text{L-Glutamate} + NH_3 \qquad (4)$$

$$N_1TP + N_2DP \underset{\text{Glutamate, Glutamine}}{\overset{Me^{2+},\ P_i \text{ or } As_i}{\rightleftharpoons}} N_2TP + N_1DP \qquad (5)$$

The abbreviation $Me^{2+}$, $As_i$, $P_i$, $N_1$ and $N_2$ represent divalent metal ions, arsenate, orthophosphate, nucleoside 1 and nucleoside 2, respectively. The enzymic activities which catalyze the various reactions are referred to as follows: reaction (1), biosynthetic activity, reaction (2), transferase; reaction (3), ATPase; reaction (4), arsenate dependent glutaminase; and reaction (5), transphosphorylase.

The proposed mechanistic scheme is: (The parenthesis indicates enzyme bound complex.)

Glut + ATP
Pyrrolidone Carboxylate + $P_i$ + ADP
I
II
III
IV
V
$NH_2OH$
$NH_3$
$NH_2OH$
$NH_3$
GluNOH + $P_i$ + ADP
GluNOH + $P_i$ + ADP
Gln + $P_i$ + ADP
Gln + $P_i$ + ADP

Evidence for supporting the above mechanistic scheme are: (i) Glutamate for reactions (1) and (3), and glutamine for reactions (2) and (4) occupy the same binding site. (ii) ATP for reactions (1) and (3), and ADP for reactions (2) and (4) occupy the same site. (iii) Orthophosphate and arsenate bind at a site which overlaps the $\gamma$-phosphate site of nucleoside triphosphate. (iv) Both $Mg^{2+}$ and $Mn^{2+}$ support the formation of reaction intermediate observed in reactions (1) and (3). In addition they both support the reverse biosynthetic reaction. (v) Observed transphosphorylation between $N_1TP$ and $N_2DP$ is consistent with the coupling of both forward and reverse biosynthetic reactions. (vi) $^{18}O$ transfer is reversible between orthophosphate and $\gamma$-acyl group of glutamate. (vii) Arsenate, a better nucleophile than $P_i$ functions as a more effective substrate for the transferase reaction. However, ATP derived from $P_i$ and ADP is more stable than the corresponding arsenate derivative, therefore $P_i$ is a better substrate than arsenate for the reverse biosynthetic reaction.

In the proposed scheme, two possible mechanisms are included. One involves the formation of $\gamma$-glutamyl phosphate (or arsenate) (V) as a required intermediate for the biosynthetic reaction, and the other involves partial bond breaking and forming transition state intermediates such as I, II, and III. Both mechanisms require the interaction of $P_i$ (or $As_i$) oxygen with the $\delta$-carbon of glutamine for initiating the reverse biosynthetic and transfer reactions; and the interaction of $\gamma$-acyl oxygen of glutamate with the $\gamma$-phosphorus of nucleoside triphosphate for forward biosynthetic and glutamate dependent ATPase reactions. The mechanism which requires $\gamma$-glutamyl phosphate as an intermediate implies that in the reverse biosynthetic and transfer reactions, ADP does not interact directly with $P_i$ (or arsenate) to form ATP; instead, ADP is utilized for inducing (or stabilizing) the protein conformation needed, and provides free energy for the formation of $\gamma$-glutamyl phosphate. A direct ADP-$P_i$ interaction in the reverse biosynthetic reaction would proceed by III $\rightarrow$ II $\rightarrow$ V $\rightarrow$ I which means that the forward biosynthetic reaction will proceed by I $\rightarrow$ V $\rightarrow$ II $\rightarrow$ III. In other words, the $NH_3$ addition step would be II $\rightarrow$ III, which is inconsistent with the assumption that $NH_3$ attacks $\gamma$-glutamyl phosphate. In the second mechanism, $\gamma$-glutamyl phosphate is formed only when $NH_3$ and $NH_2OH$ are not present. With this mechanism, the biosynthetic reaction will proceed from glutamate, ATP $\rightleftarrows$ I $\rightleftarrows$ II $\rightleftarrows$ III $\rightleftarrows$ glutamine, ADP and $P_i$, while the transfer reaction pathway is glutamine $\rightarrow$ III $\rightarrow$ II $\rightarrow$ IV $\rightarrow$ $\gamma$-glutamyl hydroxamate.

2. The rates of ADP and $P_i$ binding to the unadenylylated glutamine synthetase from E. coli were studies by the stopped-flow technique.

The following six reactions were studied.

(1) ADP + EMn
(2) ADP + EMn $P_i$
(3) $P_i$ + EMn
(4) $P_i$ + EMn ADP
(5) ADP + EMg
(6) ADP + EMg $P_i$

Experimental results obtained at various substrate concentrations suggest that there are two steps involved; the first is formation of a binary complex (ES), followed by a relatively slower conformational change to ES'

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES' \qquad (6)$$

Under the psuedo first order conditions, $k_{obs}$ obtained from the analysis of the rates of the fluorescence change can be described by equation (7).

$$k_{obs} = \frac{k_2}{1 + \frac{1}{K_1[S]}} + k_{-2} \qquad (7)$$

where $K_1 = \frac{k_1}{k_{-1}}$.

Utilizing a curve fitting computer program, the following values were obtained at 15°.

| | $K_1$ | $k_2$ | $k_{-2}$ | $K_{diss}$ |
|---|---|---|---|---|
| Reaction 1 | $3.9 \times 10^4$ $M^{-1}$ | 90 $sec^{-1}$ | 9.5 $sec^{-1}$ | $3 \times 10^{-6}$ M |
| Reaction 2 | $4.9 \times 10^4$ | 220 | 0.5 | $3.5 \times 10^{-8}$ |
| Reaction 4 | 230 | 216 | 1.8 | $3.7 \times 10^{-5}$ |

$K_{diss}$ is calculated from the equation $K_{diss} = k_{-2}/K_1 k_2$.

Reaction (3), (5), and (6) are too fast and their $K_{diss}$ are too high to permit accurate measurement of the rate constant such that a concentration dependent rate profile can be established. However maximum values of $k_{obs}$ which is independent of substrate concentration, can be estimated by using our home-made fast mixing cell. The values of $k_{obs}$ are

| | $k_{obs}$ |
|---|---|
| Reaction 3 | 1000 $sec^{-1}$ |
| Reaction 5 | 1000 |
| Reaction 6 | 310 |

The values of $K_{diss}$ for reaction (1) - (6) were also measured independently by fluorescence titration method. These results are in good agreement with the $K_{diss}$ calculated from the kinetic data.

| | $K_{diss}$ |
|---|---|
| Reaction 1 | $4.6 \times 10^{-6}$ M |
| Reaction 2 | $5 \times 10^{-8}$ |
| Reaction 3 | $1.4 \times 10^{-3}$ |
| Reaction 4 | $3 \times 10^{-5}$ |
| Reaction 5 | $4 \times 10^{-5}$ |
| Reaction 6 | $3 \times 10^{-5}$ |

The rate of ADP release from the $Mn^{2+}$ activated enzyme with the presence of $P_i$ is very slow ($k_{-2}$ = 0.5 $sec^{-1}$). This slow rate is responsible for the observed inactivation of biosynthetic reaction by $Mn^{2+}$ with a conventional assay method for ADP or $P_i$ detection.

3. With the unadenylylated enzyme in the presence of $Mg^{2+}$, L-glutamine binds to the enzyme and enhances the tryptophan fluorescence of the protein. The reaction rate is very fast. Due to relatively high rate constants and high dissociation constant ($K_{diss}$ = 7 mM, obtained from the amplitude), one cannot obtain accurately the glutamine concentration dependent rate. But the concentration independent rate is 120 $sec^{-1}$, which is corresponding to the sum of the $k_2$ and $k_{-2}$ (see equation 7). In the case of $Mn^{2+}$ enzyme, the L-glutamine binding is enhanced by the presence of ADP. The enhancement factor is equal to 2 as indicated by fluorescence titration of ADP to the enzyme and enzyme-glutamine complex.

4. By following the protein fluorescence changes, due to substrate binding and overall biosynthetic reaction, we can evaluate the $k_{cat}$ and individual rate constants of the reaction process catalyzed by the $Mg^{2+}$ activated enzyme. The data indicate that two different intermediates are formed during the course of the reaction. In the absence of $NH_3$, the two intermediates, whose rates of formation are different, are revealed by the biphasic nature of the kinetic data. However, in the presence of limiting amount of $NH_3$, only the fast forming intermediate is observed. From the time required for the consumption of ammonia, one can calculate the $k_{cat}$ which is in good agreement with that obtained from steady-state data. As soon as $NH_3$ is consumed, the second intermediate is reformed. The fact that a fluorescence signal corresponding to the second intermediate was not observed in the presence of $NH_3$, is due to the rapid reaction rate of $NH_3$ with the second intermediate, as indicated by the kinetic studies of the addition of $NH_3$ to MgEATP-Glut.

5. Utilizing stopped-flow spectrometer, a technique was introduced for determining ATP concentration in either cell crude extract or purified system with luciferase-luciferin reaction. Due to the fact that light produced from luciferase-luciferin-$O_2$-ATP reaction decays rapidly, and the decay rate is shown to be ATP concentration dependent. In addition, if the sample contains ADP and other nucleoside triphosphates, it is observed that nucleoside diphosphokinase in the firefly lantern will catalyze the conversion of ADP to ATP in a relatively slower rate than the luciferin-luciferase reaction. Therefore it is necessary to capture the initial rise of light intensity produced by the luciferase catalyzed reaction. Stopped-flow spectrometer is used to record the initial light intensity. This technique is shown to be a more quantative method for ATP assay when luciferin-luciferase system is used; and with the addition of luciferin, which will increase light intensity by a factor of 50 to 100, one can measure accurately picomole amounts of ATP. In addition, the interference from other nucleotides can be detected and differentiated by the stopped-flow technique.

It is interesting to point out that first order rate is followed for at least 87% of the light producing reaction. This is the oxidation of luciferase-luciferin-AMP complex by oxygen to form oxyluciferin, AMP, and $CO_2$. The observed rate constant is $2.3 \pm 0.2$ $sec^{-1}$ at 25°. The observed rate is independent of ATP concentration from 0.5 μM to 0.1 mM range. This suggests that (a) oxygen (ca ~ 1 mM) is not limited since the amplitude is directly proportional to ATP concentration and (b) the rate determining step is a unimolecular reaction of luciferase-luciferin-AMP-$O_2$ complex to form oxyluciferin.

6. Fast reaction kinetic studies of the interaction of actin, subfractment-1 of myosin and ATP were carried out with the collaboration of Drs. Stephen Chock and Evan Eisenberg. With light scattering and fluorescence methods to detect the interaction of myosin with actin and actomyosin with ATP respectively, it is possible to follow the whole cycle for ATP hydrolysis by actomyosin. The results suggest that ATP first binds to actomyosin to initiate the dissociation of actomyosin to actin and ATP-myosin. The ATP-myosin complex then undergoes conformational changes which accompanied by partial hydrolysis of ATP. Complete ATP hydrolysis was accomplished when actin rebind to the myosin.

Significance to Bio-Medical Research:

The aim is to gain better understanding of how enzymes function with respect to its catalytic and regulatory properties.

Proposed Course of Research:

1) To further improve the existent machines with respect to their sensitivity and shortening the dead-time of the stopped-flow machine. In addition, to build a laser heating temperature-jump machine and a slow heat exchange temperature-jump machine to cover the nanosecond and second range respectively.

2) To further explore the physical and chemical properties of the unadenylylated and adenylylated glutamine synthetase with respect to their catalytic and regulatory functions. In particular, we will utilize the stopped-flow technique to study the kinetics of inhibition and catalysis at high enzyme level such as that present in vivo.

3) To study the kinetic and mechanism of DNA-repressor interaction utilizing the tryptophan fluorescence of the repressor.

Keyword Descriptors:

Glutamine synthetase, steady-state kinetics, fast reaction kinetics, transient kinetics, mechanistic study, luciferin-luciferase, actin, actomyosin.

Honors and Awards: None

Publications:

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6. P. Hambright, M. Krishnamurthy, and P. B. Chock: Metal-Porphyrin Interactions. V. Kinetics of Cyanide Addition to a Water Soluble Iron Porphyrin Dimer. J. Inorg. Nucl. Chem. 37: 557-561, 1975.

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Project No. Z01 HL 00203-02 LB
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Cellular Regulation of Enzyme Levels

Previous Serial Number: NHLI-2

Principal Investigator: Donald M. Pehlke

Other Investigators: E. R. Stadtman

Cooperating Units: None

Project Description:

Objectives: Control of cellular enzyme concentration is an important mechanism of metabolic regulation. The level of any functional protein reflects the balance between its de novo synthesis and its degradation. While investigations of the process by which proteins are synthesized have yielded important understandings directly relevant to biomedical applications (e.g., mechanisms of hormone action, antibiotics), comparatively little is known about the process by which proteins are degraded. Since metabolic energy, RNA synthesis, and protein synthesis are required for protein degradation, the probability that the process plays an important metabolic role is high.

In procaryotes, the transition from the logarithmic phase of growth to stationary phase provokes the rapid loss of certain enzymes and an increase in protein degradation as measured by the release of radio-labelled amino acids from pre-labelled proteins. To date, this process has required the intact cell in E. coli. Studies from this laboratory of alterations in fifteen different enzymes in E. coli led to the observation that significant losses of enzymatic activity occurred with threonine-sensitive aspartokinase, lysine-sensitive aspartokinase, and homoserine dehydrogenase. These are key enzymes involved in the synthesis of the aspartate family of amino acids. The loss of these activities, therefore, represents a significant and rational metabolic adaptation to nitrogen starvation. These enzymes were selected for further detailed study of the biochemical mechanisms underlying their degradation as a possible model with which to investigate the regulation of the protein breakdown system.

Major Findings:

I. Whole cell studies: A system was developed to assess the above enzyme activities in intact cells of E. coli W using frozen-thawed cells briefly exposed to toluene which renders the cell membrane permeable to small mole-

cules. Inactivation of the lysine sensitive aspartokinase (Lys AK) is not affected by 2,4 dinitrophenol but is blocked by anaerobic conditions or cyanide under standard conditions of nitrogen starvation. A carbon source (glucose or glycerol) is required. No inactivation is observed in the absence of divalent cations. Loss of Lys AK activity is blocked by inhibition of protein synthesis with chloramphenicol. Phenylmethanesulfonylfluoride (PMSF), an inhibitor of serine proteases, has no effect. Amino acid end products of the aspartate pathway did not affect Lys AK inactivation in the intact cell.

Threonine sensitive aspartokinase (Thr AK) inactivation in responce to nitrogen starvation was also studied with the above effectors. 2,4 DNP has no effect while $O_2$ deprivation and exposure to KCN block inactivation. EDTA and chloramphenicol prevent activity loss; PMSF is not effective. A carbon source seems to be required. Threonine partially blocks Thr AK inactivation while lysine increases the rate of loss.

Observations on the homoserine dehydrogenase activity (HSDH) qualitatively paralleled the Thr AK findings. Since both enzymatic activities are present on the same polypeptide in E. coli, this is not surprising. However, quantitative differences between the two activity losses were noted which suggest interesting regulatory possibilities.

II. Cell free studies: A cell free extract capable of inactivating partially purified Thr AK and HSDH but not Lys AK has been discovered in stationary phase cells. This inactivating activity is enzymatic in nature and requires an activator or co-factor present in boiled cell extracts. The activator has been purified and identified from boiled cell extracts. Purification of the protein responsible for inactivating activity is in progress.

Relevance to biomedical research:

While it is difficult to estimate the impact of the elucidation of a basic cellular process, an understanding of the mechanisms and regulation of protein degradation would have broad application in biochemistry, bacteriology, and medicine. In bacteria, the probability that protein degradation is necessary for successful adaptation to new cellular environments might be exploited in the design of new antibiotics.

Studies of mammalian muscle have demonstrated that protein degradation is central to the negative nitrogen balance and muscle atrophy associated with disuse. Similar questions must be asked about the role of protein breakdown in situations such as wound healing, anoxic myocardial injury, and diabetes. Better understanding of the proteolytic enzymes of the coagulation, complement, and kinin systems may arise from the study of protein degradation.

Nutritional disorders and aging are more directly related to the subject of this report. Evidence is accumulating that there is an increase in functionally abnormal proteins with age. The fate of these proteins, the metabolic significance of their presence, and the question of whether their presence

reflects an altered degradation system relate to a basic understanding of the aging process. Similarly, the study of the nitrogen starved system discussed in this report may lead to new insights about the processes involved in protein malnutrition in man.

Finally, while there are no presently known genetic disorders thought to be due to defective protein degradation, there are many examples of cross reacting material (CRM) negative enzyme deficiency states. These have been interpreted as deficiencies of de novo synthesis. Many could alternatively be interpreted as defects in control of the degradative process with increased loss of the protein of interest.

Proposed Course of Action:

Purification of the Thr AK/HSDH inactivating protein will continue with the hope of attaining a homogeneous preparation with which to study the mechanism by which AK activity is lost. It will be necessary to determine whether the loss of activity is due to enzyme modification or proteolytic cleavage, whether the reaction is specific for Thr AK or is generalized, how the reaction is controlled, and how it relates to the regulation of cell metabolism. In addition to enzymologic and physical approaches, antibodies will be raised to both the Thr AK/HSDH and to the inactivating protein in order to study their interaction immuno-chemically. Efforts at isolating a genetic mutant lacking the inactivating activity will be made.

Keyword Descriptors:

E. coli, threonine sensitive aspartokinase, lysine sensitive aspartokinase, homoserine dehydrogenase, protein degradation.

Honors and Awards: None.

Publications: None.

Project No. Z01 HL 00210-02 LB
1. Laboratory of Biochemistry
2. Section on Enzymes
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Regulation of the Cascade Control of E. coli Glutamine Synthetase

Previous Serial Number: NHLI-1

Principal Investigator: Edgar G. Engleman
Sharron H. Francis

Other Investigator: Earl R. Stadtman

Cooperating Units: None

Project Description:

Objectives: In addition to regulation by cumulative feedback inhibition, glutamine synthetase from E. coli W is regulated by the covalent attachment of an AMP moiety to a tyrosyl residue in each of the enzyme subunits. The attachment and removal of the AMP is catalyzed by an enzyme known as adenylyltransferase (ATase). The activity of ATase is modulated, however, by a second protein, $P_{II}$, which exists in two forms. $P_{IIA}$ and $P_{IID}$ promote adenylylating activity and deadenylylating activity of the ATase, respectively. $P_{IIA}$ is converted to $P_{IID}$ by the covalent attachment of a UMP group to a tyrosine in each of its subunits. The interconversion of $P_{IIA}$ and $P_{IID}$ is catalyzed by the uridylyltransferase and uridylyl-removing enzyme(s).

Major Findings:

In our studies we have examined the effects of a number of metabolites on the respective enzymatic steps in the cascade control of E. coli glutamine synthetase. The ATase, $P_{IIA}$ and $P_{IID}$ and the uridylyl transferase·uridylyl removing enzyme(s) were isolated for these studies and the role of effectors in each step examined.

The complex control of the cascade is evidenced by the large number of substances which exert potent inhibitory or stimulatory effects at physiological levels. The reciprocal effects of glutamine and α-ketoglutarate are seen at every step, suggesting not only an important regulatory function for these two compounds, but also a specific binding site for these ligands on the respective proteins.

The $P_{IIA}$ supported adenylylation of glutamine synthetase by the adenylyl transferase is inhibited by the addition of either $P_{IID}$ or α-ketoglutarate. However, α-ketoglutarate and $P_{IID}$ in combination act synergystically to in-

hibit this activity. Of the other substances tested, only methionine and tryptophan stimulated adenylylation at physiological levels, and this stimulation appears to be mediated by a direct effect on ATase and is not dependent on the $P_{IIA}$-ATase complex. At high (12mM) levels coenzyme A and 3-phosphoglycerate inhibit adenylylation.

The $P_{IID}$ supported deadenylation requires the presence of α-ketoglutarate and ATP and the level of α-ketoglutarate required for activity is closely related to the level of ATP. At 0.03 mM α-ketoglutarate and 2.5 mM ATP, $P_{IID}$ supported deadenylation is 80% of the maximal activity. Under these conditions glutamine inhibits deadenylation but $P_{IIA}$ has little or no effect. However, the combination of $P_{IIA}$ and glutamine produce a greater inhibition than glutamine alone, suggesting synergism analogous to $P_{IID}$-α-ketoglutarate inhibition of adenylylation. At high concentrations (10 mM) a variety of metabolites inhibit the deadenylation, i.e., CoA, UTP, 3-phosphoglycerate, phosphoenol pyruvate, fructose 1,6-diphosphate, and tryptophan. However, at 1 mM concentrations only UTP, PEP, and 3-PGA show any effects.

A search for endogenous inhibitors of the UR enzyme seemed warranted by the observation that this activity could not be assayed in the crude homogenate from E. coli. Furthermore, addition of E. coli crude extract to partially purified UR effectively inhibited its activity. Two inhibitors, CMP and UMP, were identified from these E. coli extracts in the following manner. Passage of a streptomycin supernatant over Sephadex G-200 yielded a large inhibitor complex which when heated at 60° C for 30 minutes dissociated into an active dialyzable fraction that was adsorbed to charcoal and further separated over a Dowex 50 column. Thin-layer chromatography yielded two active moieties, which were identified as CMP and UMP on the basis of $R_f$ values and UV spectra.

The existence of a large relatively specific binding substance for CMP and UMP is demonstrated by the isolation of the inhibitors from the void fraction from a G-200 column. Radioactively labeled CMP and UMP readily exchange into this fraction but TMP, IMP, and AMP are bound to a lesser degree. Further characterization of this binding substance has been difficult because it is resistant to proteases, nucleases, phospholipases and lysozyme.

Although all nucleotides directly tested inhibited the Mn supported UR activity the monophosphates were at least one order of magnitude greater in potency than the diphosphates which were more potent than the triphosphates. Of the other substances tested coenzyme A was the most potent inhibitor of UR activity. None of these inhibitors of Mn-supported UR had any effect on the UR activity supported by Mg·ATP·α-KG UR-activity, suggesting that the two UR activities may involve different mechanisms. Nonetheless, both activities were stimulated by glutamine.

Unlike UR which is unaffected by α-ketoglutarate, the UTase requires α-ketoglutarate for activity; 0.1 mM α-ketoglutarate supports 65% of the maximal activity and the UTase is potently inhibited by glutamine. For example, at 0.1 mM α-ketoglutarate and 0.1 mM glutamine the UTase activity is approximately 60% inhibited. Like the Mg·ATP·α-ketoglutarate UR-activity, UTase was unaffected by nucleotide monophosphates, coenzyme A and other metabolites tested, i.e., phosphoenol pyruvate, fructose 1,6 diphosphate, 3-phosphoglycerate, tryptophan, glycine, histidine, DPN, TPN.

Thus, all steps in the cascade control are sensitive to the metabolites primarily involved in the glutamine synthetase reaction; i.e. glutamine and α-ketoglutarate. In addition, a number of other metabolites affect the various steps in the cascade but the physiological significance of these effects are not fully understood.

Proposed Course of Project:

1. Further attempts to purify UR-UTase using techniques such as CMP/UMP affinity chromatography, and if successful, physical characterization of the enzyme.

2. Characterization of the CMP/UMP binding substance and studies regarding its possible interactions with the UR enzyme.

Keyword Descriptors: Glutamine Synthetase, Regulation, Cascade Control.

Honors and Awards: None

Publications: None

Project No. Z01 HL 00211-02 LB
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Title: Regulation of Glutamine Synthetase

Previous Serial Number: NHLI-3

Principal Investigators: Robert Park
Pauline Smyrniotis

Other Investigators: E. R. Stadtman

Cooperating Units: None

Project Description:

Objectives: 1) Using unadenylylated glutamine synthetase in the presence of $Mg^{++}$, to examine the effect of substrate and effector concentrations upon the catalytic constants of each of the substrates in the $\gamma$-glutamyl transfer reaction. 2) Through a kinetic analysis, to determine if certain end products of glutamine metabolism achieve inhibition by reacting at a common site or at multiple sites on glutamine synthetase.

Major Findings:

1. Effect of substrate concentration on catalytic constants.

$$\text{Glutamine} + NH_2OH \xrightarrow{\text{ADP Arsenate } Mg^{++}} \gamma\text{-glutamylhydroxymate} \qquad (1)$$

The apparent Michaelis constants (app. $K_m$) for glutamine, ADP, and arsenate in the transfer reaction (Reaction 1) are highly dependent upon the binding of each of the other reactants. If the arsenate concentration increases from 5 mM to 20 mM, the app. $K_m$ decreases from 14 mM to 10 mM for glutamine, and from 175 μM to 50 μM for ADP. Likewise, if ADP is gradually saturated, the app. $K_m$ decreases from 69 mM to 6 mM for arsenate, and from 18 mM to 10 mM for glutamine. As glutamine is saturated, the app. $K_m$ for arsenate decreases from 11 mM to 7 mM. This pattern of decreasing app. $K_m$ as a result of gradual substrate saturation suggests that the substrate binding of the transfer reaction is random. Further kinetic studies with respect to hydroxylamine and $Mg^{++}$ are in progress to resolve this issue.

2. Mechanism of cumulative feedback inhibition. We have continued to explore the mechanism of feedback inhibition of glutamine synthetase by alanine, AMP, histidine, and tryptophan by utilizing graphical procedures of Cleland (1) and Yagi and Ozawa (2). Inhibition constants were obtained for



6K

ATP, CTP, AMP, histidine, tryptophan, and alanine. Thereafter, by following enzyme activity and varying one inhibitor ($I_1$) at fixed levels of a second inhibitor ($I_2$), an interaction coefficient, $\alpha$, can be obtained from the graphical plots which reflects whether $I_1$ and $I_2$ compete for the same site or occupy separate sites on the enzyme. Using this approach, it was found that ATP and CTP have a common site, and AMP, histidine, and tryptophan each have separate sites. These findings were further supported by results of the graphical method of Yagi and Ozawa (2) in which plots of 1/V versus increasing concentration of 2 inhibitors varied at constant molar ratio yield straight lines for 2 inhibitors occupying the same site. Data obtained for alanine reveal conflicting results from the two different graphical methods, and this is currently being resolved. Fractional inhibition plots for AMP and alanine show that they are complete inhibitors, whereas histidine achieves only about 50% inhibition at infinite concentrations.

Significance to Bio-Medical Research:

The mechanism of enzyme regulation through feedback inhibition is the basis of certain metabolic diseases such as hypercholesterolemia, and together with other mechanisms to regulate enzyme activity, constitutes the means by which a multitude of metabolic pathways are controlled. Because of its key role in bacterial nitrogen metabolism, glutamine synthetase serves as a unique model to elucidate principles of metabolic regulation.

Proposed Course of Research:

Studies will be completed on the effect of substrate binding upon the app. $K_m$ for the transfer reaction of glutamine synthetase. In addition, kinetic studies will be continued to further elucidate the mechanism of feedback inhibition and explore the relationship between binding sites of each of the inhibitors of glutamine synthetase.

Keyword Descriptors:

Feedback inhibition, glutamine synthetase, kinetics, catalytic constants, inhibitors.

Honors and Awards: None

Publications:

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Project No. Z01 HL 00212-04 LB
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

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Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Biochemical Genetics of $NH_3$-Assimilatory Enzymes in E. coli $K_{12}$

Previous Serial Number: NHLI-5

Principal Investigator: Mary Anne Berberich

Other Investigators: None

Cooperating Units: Dr. J. Phillips, Section on Laboratory Animal Medicine and Surgery, National Heart and Lung Institute, NIH

Project Description:

Objectives: 1) To isolate and characterize biochemically, mutants in which the activity of glutamine synthetase, glutamate synthase or glutamate dehydrogenase is affected and to conduct genetic experiments for the purpose of locating these mutations on the E. coli chromosomal map. 2) To develop methods to study the physiological inter-relationship of the $NH_3$-assimilatory enzymes in E. coli.

Major Findings:

Earlier work from this laboratory determined that the genes designating glutamine synthetase (GS), glutamate synthase (GAT), and glutamate dehydrogenase (GDH) do not constitute a contiguous operon in E. coli and may be located at approximate map positions 77', 50', and 20', respectively. Enzymic analysis of a group of revertants from $gln^-$ to $gln^+$ revealed a thermolabile GS in each case thereby substantiating that the gene locus at 77', gln A, is the structural gene for GS. Continued investigation suggested that these enzymes might be involved in a more subtle scheme relating the regulation of amino acid metabolism and $NH_3$ assimilation.

Two of the revertants with thermolabile GS exhibited poor derepression on limiting $NH_3$ although the adenylylation values for the GS were low as expected. In order to reconcile these data with the autogenous regulation scheme proposed by Magasanik et al. the scheme should be expanded to include a positive activation by GS as well as repression by adenylylated GS. Derepression on limiting-$NH_3$ and derepression on glutamate are probably related to an increase in intracellular concentration of $\alpha$-ketoglutarate. One of the two revertants described above was also unable to derepress when grown on glutamate whereas the other derepressed to approximately 30% wild-type extent. These results may indicate involvement of an additional component in

derepression which can only be discerned from the pattern of regulation of the altered revertant enzyme. This is being examined further.

Preliminary evidence from another laboratory suggests that constitutivity of GS can result from mutations affecting enzymes involved in the chain of biochemical events leading to the modification of GS by adenylylation (Abstr. of Ann. Meeting of ASM, New York, 1975). It is interesting that the genes gln B and gln D, apparently designating $P_{II}$ regulatory protein and uridylating enzyme have been tentatively assigned map positions approximating that for GAT (50'). Another locus, gln E, the apparent determinant for uridylyl removing enzyme is designated in the general map region of GDH ($\sim$20'). This genetic juxtaposition may be significant in the scheme of regulation of $NH_3$ assimilation and is being examined further.

Growth in a minimal salts medium containing glucose at 33 mm and glutamate at 60 mm gives optimal derepression of GS. This is supplemented where necessary with the amino acids threonine, leucine, histidine and arginine at 100 µg/ml which collectively show no repressive effects, e.g. via $NH_3$ contribution, in wild-type. Under these conditions, (with or without amino acid supplement) the activity of GAT is almost completely repressed and there is a marked elevation in the level of GDH activity. The extent of derepression appears independent of the state of adenylylation of GS. Also, there is no corroboration in E. coli of the observation reported for Klebiella that a reciprocal relationship exists between GS and GDH. According to Brenchley (personal communication) studies with S. typhimurium agree with the E. coli findings. Furthermore, there is no evidence in either coli or typhimurium for $NH_3$ induction of GDH. In wild-type E. coli, the activities of both GDH and GAT may be decreased to $\sim$ 50% original value by increasing $NH_3$ concentration to 100 mM. These differences may reflect the adaptive changes dictated by the milieu of soil-living vs. enteric organisms.

Some recent findings with a temperature-sensitive glutamate t-rna synthetase mutant indicate that, at the restrictive temperature, the activity of GAT is elevated as is that of GS (La Pointe, J., personal communication). The interpretation offered is that charged glutamyl-t-rna is a co-repressor for both GS and GAT. However the observations of repressed GAT with derepression of GS on high glutamate reported here do not agree with the idea of unity in control for these two enzymes. The relationship between GAT and GS is currently being investigated from another point of view (see last section below).

Attempts to correlate the phenomenon of MSO resistance with the mechanism of GS derepression are in progress and early work suggests that, if the structural gene for GS is autogenously regulated, it functions via a cooperation with some other element - perhaps a component of GAT. In this regard, it is interesting that, in the case of Klebsiella, the gln $C^-$ regulatory type mutations which are now assigned positions within the gln A structural gene locus, were originally derived from a $GAT^-$ parent. The observation that methionine potentiates MSO resistance is being investigated in view of the fact that methionine is a potent inhibitor of GAT. Methionine

has also been observed to reduce the growth rate of wild-type cells in unsupplemented minimal medium.

Antibody has been prepared in the rabbit against purified GAT. Although no cross-reactivity could be observed between $p_{II}$ A or D; GS $n_0^-$ or $\bar{7}$ and anti-GAT antiserum via a micro precipitin test, approximately 35% neutralization of enzyme activity could be observed when anti-GAT serum was added to $GS_{\overline{II.7}}$ prior to transferase assay. At this antiserum concentration the activity of the homologous antigen (GAT) was 68% inhibited. Control serum did not inhibit and GS $\bar{n}$ 1.0 appears unaffected. Some inhibition of $p_{II}$ activity ($\sim$15%) was observed using very small amounts of antiserum however with larger amounts the control serum showed inhibition. These results are preliminary and the antiserum will be fractionated in an attempt to eliminate non-specific inhibition. Sub-unit sharing among the $NH_3$ assimilatory enzymes may represent an additional regulatory opportunity.

Proposed Course of Project:

Genetic studies to refine the chromosomal regions containing the structural genes for glutamine synthetase, glutamate dehydrogenase, glutamate synthase will continue with an emphasis on isolation of regulatory mutants. Enzymic analysis of revertants of structural gene mutants will continue. Physiological and biochemical studies on the interrelationships of $NH_3$-assimilatory enzymes in mutant strains will continue.

Keyword Descriptors:

Genetics of glutamine synthetase, regulation of $NH_3$ metabolism, drug resistance and derepression of glutamine synthetase.

Honors and Awards: None

Publications: None

Project No. Z01 HL 00213-01 LB
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Title: Metabolite Regulation of Coupled Covalent Modification Cascade Systems

Previous Serial Number: NHLI-3

Principal Investigator: E. R. Stadtman

Other Investigators: P. B. Chock
S. P. Adler

Cooperating Units: None

Project Description:

Objectives: To make a theoretical analysis of the steady state functions involved in the allosteric regulation of key enzymes in metabolism by cascades of cyclic covalent modification reactions.

Major Findings:

Regulation of several key enzymes in metabolism is mediated by cyclic covalent modification reactions in which the active form of an enzyme in one cycle is a catalyst for the covalent modification of an enzyme in the next. When the cascade involves (n-1) successive cyclic covalent modification reactions and an allosteric activation of the first enzyme in the series, it can be shown that under steady state equilibrium conditions, the fraction of the modified form of the target enzyme ($E_{na}$) is described by Eq. (1),

$$\frac{E_{na}}{E_n} = \frac{1}{\left(\frac{K_d}{e} + 1\right)\left(\frac{k'_r}{k'_f}\right)^{n-1} + \left(\frac{k'_r}{k'_f}\right)^{n-2} + \cdots \frac{k'_r}{k'_f} + 1} \qquad (1)$$

with the assumptions that: (1) the catalytic constants for the forward step, $k'_f = k_{1f} E_1 = k_{2f} E_2 = \cdots k_{(n-1)f} E_{(n-1)}$, where $k_{1f}$, $k_{2f}$, $\cdots k_{(n-1)f}$ are specific rate constants for successive forward steps in the cycles and $E_1$, $E_2$, $\cdots E_n$ are total concentrations of enzymes undergoing activation at the first step, first cycle and (n-1) cycle respectively; (2) the catalytic constants for the regeneration of the unmodified forms, $k'_r = k_{1r} R_1 = k_{2r} R_2 = \cdots k_{(n-1)r} R_{n-1}$, where $k_{1r}$, $k_{2r}$, $\cdots k_{(n-1)r}$ are specific rate constants for the successive regeneration steps and $R_1$, $R_2$, $\cdots R_{(n-1)}$ are concentrations of enzymes catalyzing the regeneration steps. Equation (1) demonstrates the tremendous amplification potential of such cascade systems. It follows that

the concentration of effector, e, required to produce 50% conversion of $E_n$ to $E_{na}$, decreases in such a manner that log $e_{0.5}$ is inversely proportional to the number of cycles in the cascade. Thus, when $K_d$ = 1 mM and $k_r'/k_f'$ = 0.1, $e_{0.5}$ is approximately equal to 1, 0.1, 0.01 and 0.001 mM for a 0, 1, 2, and 3 cycle cascade, respectively. In addition to this amplification capacity, such systems exhibit an enormous capacity for allosteric control. It is evident from Eq. (1) that positive or negative allosteric interactions with any one or all of the several enzymes in the cascade will affect the catalytic constants of these enzymes and thereby regulate the over-all ratio of $k_r'/k_f'$, which in turn determines the degree of amplification.

The foregoing cascade model is patterned after the system that regulates the activities of phosphorylase or glycogen synthetase in which a series of protein kinases and phosphatases catalyze the phosphorylation and dephosphorylation of the target enzymes. A somewhat different kind of cascade regulates the activity of glutamine synthetase in E. coli. Here, regulation is mediated by the coupling of two cyclic nucleotidylylation processes. The first cycle consists of uridylylation and deuridylylation of the $P_{II}$ regulatory protein. This is catalyzed by a protein complex exhibiting both uridylyltransferase (UTase) and uridylyl removing (UR) activities, and involves the attachment and removal of a uridylyl group to and from a tyrosyl hydroxyl group in each of four identical subunits of the regulatory protein. A second cycle, involves adenylylation and deadenylylation of a tyrosyl hydroxyl group in each of the 12 subunits of glutamine synthetase (GS), and is catalyzed by adenylyltransferase (ATase). Coupling of the two cycles is obtained by almost complete dependence of GS-adenylylation on the unmodified form ($P_{IIA}$) and of GS-deadenylylation on the uridylylated form ($P_{IID}$) of the regulatory protein. Because the two interconvertable forms of $P_{II}$ are oppositional in their capacities to stimulate the Atase catalyzed adenylylation and deadenylylation reactions, the steady state level of adenylylated GS is given by the expression,

$$GS_n = \frac{12\ k_1 k_3 U_R}{k_2 k_4 U_T + k_1 k_3 U_R}$$

where n = the average number of covalently bound adenylyl groups per mole of GS; $k_1$, $k_2$, $k_3$, and $k_4$ are the specific rate constants for the deuridylylation, uridylylation, adenylylation and deadenylylation reactions, respectively; and, $U_T$ and $U_R$ are the concentrations of UTase and UR removing activities, respectively. Variations in any one or all of these parameters, in response to fluctuations in the relative concentrations of allosteric effectors (viz, UTP, ATP, CMP, UMP, glutamine, $\alpha$-ketoglutarate, Pi, $Mg^{2+}$ and $Mn^{2+}$) lead to the establishment of different steady state levels of GS adenylylation and hence its catalytic activity. In the above derivations it is assumed that the affinities of the various modifying enzymes for their protein substrates is relatively small; i.e., that protein-protein interactions do not affect significantly the concentrations of the various enzyme forms. If the affinities are high, then preferential binding of one catalyst to the modified or unmodified forms of its substrate could result in deviations from the theoretical relationship described. Clearly in the above cascade systems, the

coupling of nucleoside triphosphate dependent covalent modification reactions with regeneration of the unmodified enzyme forms, results in the net decomposition of ATP and UTP. It is assumed that the concentrations of ATP and UTP are constant for a given metabolic state. Nevertheless, the apparent loss in ATP energy accompanying the cyclic processes is not wasted, this energy is used to provide the driving force that is needed to maintain the modified and unmodified forms of the various enzymes at metabolite specified steady state levels that are away from true thermodynamic equalibrium values. The consumption of ATP energy is therefore the price that must be paid to support the elegant cascade type of cellular regulation. In the last analysis cascade systems represent physiological computers, the circuitry of which consists of a series of interconnected terminals in the form of interconvertable enzymes. By means of allosteric and active site interactions, these interconvertable enzymes are programmed to sense fluctuations in the concentrations of a multiplicity of metabolites; this leads to automatic adjustments in the specific activities and rate constants of the several cascade enzymes. Through this system the multiple inputs are integrated and registered as a single output; i.e., the fractional modification of the target enzyme which ultimately determines its catalytic activity.

Proposed Course of Research:

The theoretical steady state analysis of cascade systems will be extended to include a consideration of the effects of protein-protein interactions and also to evaluate the expenditure of ATP energy needed to maintain steady states away from thermodynamic levels. An attempt will be made to determine the specific rate constants for the reaction involved in the glutamine synthetase cascade in order to test the validity of the theoretical steady state analysis.

Keyword Descriptors:

Cascade regulation, metabolic regulation, covalent modification, glutamine synthetase system, adenylylation-deadenylylation, and uridylylation-deuridylylation.

Honors and Awards: None

Publications:

1. S. P. Adler and E. R. Stadtman: Cascade Control of E. coli Glutamine Synthetase. In Richter, D. (Ed.): Lipmann Symposium. Energy, Regulation and Biosynthesis in Molecular Biology. Walter de Gruyter, Berlin-New York, 1974, pp. 28-39.

2. E. R. Stadtman, J. E. Ciardi, P. Z. Smyrniotis, A. Segal, A. Ginsburg, and S. P. Adler: Role of Adenylylated Glutamine Synthetase Enzymes and Uridylylated Regulatory Protein Enzymes in the Regulation of Glutamine Synthetase Activity in Escherichia coli. In Market, C. L. (Ed.): Isoenzymes II: Physiological Function. New York, Academic Press, 1975, pp. 715-732.

3. R. E. Miller, E. Shelton, and E. R. Stadtman: Zinc Induced Paracrystalline Aggregation of Glutamine Synthetase. Arch. Biochem. Biophys. 163: 155-171, 1974.

4. A. Ginsburg and E. R. Stadtman: Glutamine Synthetase of Escherichia coli: Structure and Regulation. In Ebner, K. E. (Ed.): Subunit Enzymes: Biochemistry and Function. New York, M. Dekker, Inc., 1975 (in press).

Project No. Z01 HL 00214-01 LB
1. Laboratory of Biochemistry
2. Section on Enzymes
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Enzyme Degradation in Klebsiella aerogenes

Principal Investigator: Richard M. Fulks

Other Investigators: Earl R. Stadtman

Project Description:

The biochemical mechanisms involved in protein breakdown and its regulation, and the role protein breakdown plays in controlling the amount of a protein present within a cell.

Major Findings:

Klebsiella aerogenes, an organism closely related to E. coli, and one that has proved useful in many biochemical and genetic studies was selected as experimental material. Preliminary experiments showed that the aspartokinase activity of this organism began to fall within minutes of onset of stationary phase due to nitrogen limitation. Within three hours, over half of the activity present at the beginning of stationary phase was lost. At the same time glutamine synthetase activity increased in these nitrogen-limited cells and this showed that the inactivation process was specific in nature. Aspartokinase inactivation also occurred in cell suspensions and this meant that large volumes of cells could be grown at once and stored frozen, then small portions of cells thawed and used for individual experiments as needed.

Addition of glucose promoted the inactivation process and dinitrophenol caused reduction in the rate of inactivation. These findings are consistent with an energy-dependent inactivation process, as has been proposed for most instances of intracellular protein degradation. Chloramphenicol, an inhibitor of protein synthesis, caused a more rapid loss of aspartokinase activity. This result suggested that in the absence of inhibitor, the cells were synthesizing new enzyme, but they were inactivating it at a rate that exceeded synthesis. As was true in the absence of chloramphenicol, dinitrophenol inhibited inactivation of the enzyme when chloramphenicol was present.

Glutamine synthetase activity also declined when chloramphenicol was present, in contrast to the rise in activity that occurred when these nitrogen-limited cells were incubated in the absence of chloramphenicol. As was true for aspartokinase, the inactivation of glutamine synthetase in the presence of chloramphenicol was inhibited by dinitrophenol. When dinitrophenol

alone was added to the medium, the level of glutamine synthetase remained nearly constant throughout 5 hours of incubation.

Study of the inactivation of glutamine synthetase offered several advantages over study of aspartokinase inactivation. One important advantage was that antibody to the E. coli glutamine synthetase had already been prepared in this laboratory by Tronick et al. and this antibody cross-reacted with the Klebsiella enzyme. Incubation of Klebsiella cells in the presence of chloramphenicol resulted in a loss of antigenic material which probably reflects intracellular degradation of glutamine synthetase. Control experiments showed that chloramphenicol did not simply interact directly with the enzyme to denature it. These findings are consistent with the interpretation that in the presence of chloramphenicol, the amount of glutamine synthetase present declined because the cells degraded the enzyme but were unable to synthesize new enzyme.

Inactivation of glutamine synthetase by covalent attachment of adenylyl groups to the enzyme affords one important way for controlling glutamine synthetase activity in these cells. However, adenylylation was not responsible for the changes observed in the present experiments, because the assays used to measure catalytic activity were equally sensitive to adenylylated or unadenylylated enzyme, and the antibody used reacts with both forms of the enzyme. Although adenylylation by itself is unable to explain the inactivation and decomposition of glutamine synthetase in these experiments, adenylylation may nevertheless play a role. For example, modification of glutamine synthetase appears to facilitate its degradation in cultured liver cells.

Proposed Research:

Experiments to further characterize the chloremphenicol-induced degradation of glutamine synthetase will be carried out. Also effects of other inhibitors such as puromycin will be examined. These studies should lay the groundwork for experiments with cell-free preparations, which can provide more precise information about the steps involved in protein degradation and its regulation. Genetic approaches may also be fruitful in the study of protein breakdown in these cells.

Keyword Descriptors:

Protein degradation, enzyme regulation, glutamine synthetase, lysine-sensitive aspartokinase, Klebsiella aerogenes, stationary phase, antibody, immunodiffusion.

Honors and Awards: None

Publications: None:

Annual Report of the
Section on Intermediary Metabolism
and Bioenergetics
Laboratory of Biochemistry
National Heart and Lung Institute
July 1, 1974 through June 30, 1975

Research in the Section on Intermediary Metabolism and Bioenergetics has been concerned with (1) the anaerobic metabolism of amino acids and other nitrogen-containing compounds with particular reference to the identification and characterization of the components of the electron transport systems linked to glycine reductase and to proline reductase, to the roles of selenium, iron, sulfur and flavins in the amino acid reductase reactions, and to the mechanism of the $B_{12}$-coenzyme dependent $\alpha$-methyleneglutarate mutase reaction (an intermediate step in the anaerobic decomposition of nicotinic acid) and (2) the metabolism of formate and other one-carbon compounds by methane-producing bacteria and the roles of selenium, molybdenum and tungsten in the methane fermentation.

Selenium Biochemistry and Anaerobic Oxido-Reduction Reactions:

In Clostridium sticklandii, Clostridium lentoputrescens and related amino acid-fermenting bacteria that utilize glycine as a terminal electron acceptor an essential component of the reductase system has been shown to be a low-molecular weight, acidic selenoprotein. The unusual ultraviolet spectrum characteristic of the oxidized form of this protein is explained by the absence of tryptophan and the presence of 5 residues of phenylalanine and 1 residue of tyrosine in the polypeptide chain. The protein contains cysteine and methionine residues and as yet unidentified selenium-containing organic compound in covalent linkage. Reduction of the protein in neutral solution with borohydride causes an instantaneous increase in absorbancy in the low ultraviolet (maximum about 238 nm) which is similar to the spectral changes observed when certain diselenides are reduced to selenols under the same conditions. Rapid reoxidation of the protein and the model compounds is indicated by immediate reversal of the spectral changes upon exposure to air. These studies, together with properties of the reduced and carboxymethylated protein, suggest that the selenium moiety is converted to a selenol (-SeH) upon reduction and the ease of reversibility of this process might explain its biological role.

In addition to greater chemical stability of the selenium moiety of the protein which is observed upon alkylation of the reduced form of the selenoprotein, both the biological activity and the antigenic specificity of the protein are lost. Specific antibodies prepared to the pure native selenoprotein fail to detect any cross-reacting precursor protein in extracts of selenium-deficient bacteria but do appear to react with a selenium-containing tryptic peptide of the native protein. Hence the sensitive immunologic approach may aid in characterization of the selenium moiety of the protein and its mode of biosynthesis.

The formate dehydrogenase of the methane-producing organism, Methanococcus vannielii, also is a selenoprotein and procedures for the partial purification of this enzyme have been developed. This complex, oxygen-sensitive enzyme is

of interest for studies as to the precise nature of oxygen sensitivity of this class of enzymes, and the role of its selenium, molybdenum and iron components. Failure of the complex form of the enzyme from the methane organism to react with antibodies to the glycine reductase selenoprotein may indicate that the chemical form of selenium in the two proteins is different but smaller molecular weight forms of the selenium-containing polypeptide will also be examined.

Proline Reductase:

The proline reductase of C. sticklandii, C. lentoputrescens and related bacteria transfers reducing equivalents to proline which, like glycine, also can serve as a terminal electron acceptor for these organisms. Proline reductase has been solubilized from the membrane of the cell by the use of detergents and purified to homogeniety by standard enzymological techniques. The pure reductase, molecular weight 298,000, consists of subunits of 61,500 and 50,500 and is a flavoprotein. Marked sensitivity of the enzyme to borohydride, hydroxylamine and other carbonyl reagents was earlier shown by Abeles et al. to be the result of modification of an essential pyruvate moiety covalently bound to the enzyme. The pure reductase is unable to react directly with the normal electron donor, a reduced pyridine nucleotide, and one or more low molecular weight carriers must be added to reconstitute the natural electron transport chain. Preliminary data showing copurification of proline reductase activity and radioactive selenium from $^{75}Se$-labeled extracts suggest that a selenium-containing catalyst may be part of the electron transport chain.

Quinone-Dependent Phosphatase of C. sticklandii:

Continued studies on the unusual mercaptan and quinone-dependent p-nitrophenylphosphatase of clostridial origin have resulted in a greatly improved method of isolation of the pure enzyme in good yield and established additional chemical properties of the protein structure. The functional form of the enzyme presumably is a quinone adduct of one or more of the four sulfhydryl groups that can be titrated on the protein. The possible function of this enzyme as a phosphorylated intermediate in an energy conservation process or in a transport process is under investigation.

α-Methylene Glutarate Mutase:

As part of a continuing investigation on the precise mechanism of the chemical rearrangements catalyzed by various $B_{12}$ coenzyme dependent enzymes, the stereochemistry of the interconversion of α-methyleneglutarate and methylitaconate by α-methyleneglutarate mutase of the nicotinate fermenting organism, Clostridium barkeri, is under study. The necessary substrates, labeled with tritium and deuterium for the determination of the stereochemical course of the reaction and with $^{14}C$ to serve as a marker of the extent of interconversion, have been synthesized by improved procedures developed especially for the problem. The necessary enzymes were prepared and better methods for assay of the reaction were developed in order that the critical experiments with the doubly labeled substrates can be carried out with precision. This type of careful study is one of the valid approaches to the elucidation of the exact mechanism of this group of poorly understood $B_{12}$ dependent reactions.

Project No. Z01 HL 00205-20 LB
1. Laboratory of Biochemistry
2. Section on Intermediary Metabolism & Bioenergetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Role of selenium in anaerobic electron transport. $CH_4$ biosynthesis.

Previous Serial Number: NHLI-13

Principal Investigator: T. C. Stadtman

Other Investigators: Belinda Seto (Staff Fellow; see individual report)
Joyce Cone (Staff Fellow; see individual report)
Raphael Martin (Guest Scientist from Spain; Spanish Fellowship; January 1975 starting date.)
Jay Jones (Technical Assistant and Predoctoral Student at George Washington U.)
Joe Nathan Davis (Research Assistant; anaerobic laboratory operator and advisor to new fellows regarding culture of microorganisms, operation of amino acid analyzers, disc gel electrophoresis equipment, etc.) See separate research report.

Project Description:

1) Anaerobic metabolism of certain amino acids with special reference to the role of selenium, quinones, flavins and non-heme iron proteins in the electron transfer and phosphorylation reactions involved. a) Structure and function of the selenoprotein component of glycine reductase and its interreaction with the other protein components of glycine reductase. Nature of the electron transfer and phosphorylation reactions linked to glycine reduction by Clostridium sticklandii and related amino acid fermenting bacteria. b) Isolation and characterization of proline reductase of C. sticklandii and identification of proteins and cofactors required for electron transport between reduced pyridine nucleotides (e.g., DPNH) and proline, the terminal electron acceptor. This project investigated primarily by Dr. Belinda Seto. 2) Mechanism of formate oxidation to carbon dioxide by Methanococcus vanielii and roles of selenium, molybdenum and vitamin $B_{12}$ in methane biosynthesis from formate. Studies to determine whether stimulation of growth of M. vannielii by tungstate is the result of substitution of tungsten for molybdenum in formate dehydrogenase. Parallel studies on formate dehydrogenase of C. sticklandii to determine the nature of the selenium-containing moiety of this enzyme, and its biochemical role. Isolation and characterization of the selenium-dependent formate dehydrogenase of M. vannielii is research project of Jay B. Jones. 3) Characterization of the quinone-dependent p-nitrophenylphosphatase

of Clostridium sticklandii (see report of J. N. Davis).

Major Findings:

1a. The chemical and physical properties of the selenoprotein (Protein A) of clostridial glycine reductase have been further characterized. Unlike one other pure selenoprotein, glutathione peroxidase, under investigation in other laboratories the glycine reductase selenoprotein is remarkably stable to a variety of chemical procedures. Only when the native selenoprotein is oxidized with peroxide or with iodine is the selenium quantitatively cleaved from the protein and lost as selenite. The protein exhibits an abnormal ultraviolet absorption spectrum; the marked fine structure in the region of 250 to 270 nm is explained by the presence in the protein of 5 phenylalanine residues and 1 tyrosine residue and the complete absence of tryptophane. Two cysteine residues are present in the protein as determined by amino acid analyses of the hydrolyzed carboxymethylated protein (2 carboxymethylcysteine residues) and by analyses of hydrolysates after performic acid oxidation (2 cysteic acid residues). Two methionine residues have been found in a number of hydrolyzed samples of the protein. The selenium containing residue in the protein is clearly distinguishable from selenomethionine and from Se-methylselenocysteine. Although the $^{75}$Se-labeled compound isolated from acid hydrolysates of the carboxymethylated selenoprotein cochromatographs in thin layer systems and on the amino acid analyzer with Se-carboxymethyl selenocysteine, it appears to be more stable than the authentic reference compound and thus its precise identity is still in doubt. The marked increase in absorbancy at 238 nm observed when the protein is reduced with borohydride at neutral pH may be attributed to the conversion of the selenium moiety to a selenol (-SeH). Model diselenides exhibit such absorbancy when reduced at neutral pH. Continued attempts to obtain a derivative of the selenocompound suitable for mass spectral analysis are in progress.

Investigations carried out by Dr. Raphael Martin to determine the amino acid composition of the amino and carboxyl ends of the selenoprotein molecule are in progress. The selenium containing moiety is located internally and is not among the amino acid residues liberated from the amino terminus by leucine amino peptidase nor from the carboxy terminus by carboxypeptidase.

Specific antibodies to the native selenoprotein were produced in rabbits and purified by Dr. Belinda Seto. Extracts and enzyme preparations that contain biologically active selenoprotein exhibit strong precipitin tests with the antibodies but no cross-reacting material was detected in selenium-deficient extracts that lack the selenoprotein activity. This suggests that either the selenium-moiety is a very important immunological determinant or synthesis of the protein ceases when selenium is unavailable to the cell. Current studies with chemically modified selenoprotein and with tryptic peptides containing the selenium moiety are in progress to aid in characterization of the selenoprotein by immunological methods.

1b. The D-proline reductase of C. sticklandii which was partially purified and studied in 1954-56, and further characterized as regards electron transport properties by Dr. Arnold Schwartz and T. C. Stadtman (1955-58) has

now been obtained in homogenous form by Dr. Belinda Seto. The pure reductase, a flavoprotein containing covalently bound pyruvate, is completely resolved of the normal electron carriers that transport reducing equivalents from reduced pyridine nucleotides (e.g., DPNH).

A large molecular weight complex of proline reductase that had been prepared earlier by Schwartz and stored at -80° still exhibited activity with DPNH and served as a source of components needed to couple the completely resolved reductase to the natural electron donor. The availability of a pure resolved terminal electron acceptor (the proline reductase) now will allow isolation and characterization of the components of this interesting anaerobic electron transport system. Earlier indications that ferredoxin and a labile protein component plus catalytic levels of acetyl-CoA were required to reconstitute the electron transport chain now can be reexamined.

2. The formate dehydrogenase of M. vannielii was purified 75-fold from crude extracts by a series of steps involving heat denaturation, ammonium sulfate precipitation and chromatography on DEAE-cellulose. All of these procedures were carried out in the absence of oxygen in the anaerobic laboratory to prevent destruction of the very oxygen-labile enzyme. The 75-fold purified enzyme preparation separated into two catalytically active bands when subjected to electrophoresis on slabs of polyacrylamide gel. Two different forms of the enzyme also were suggested by the double optimal pH profile (pH 7.5 and pH 8.5) exhibited by the purified material. Some stimulation of ability of the purified enzyme to oxidize formate with triphenyltetrazolium as electron acceptor by a low molecular weight cofactor preparation suggests the gradual separation of the enzyme from a cofactor as purification progresses.

As reported last year, evidence from growth experiments and from $^{75}Se$-labeling experiments indicates that the M. vannielii formate dehydrogenase is a selenoprotein. There is some evidence that the enzyme from other sources also may contain molybdenum and iron. The two forms of the enzyme from M. vannielii will be useful to compare analytically in view of possible differences in cofactor and electron carrier composition. Of particular importance is the identification of the oxygen-labile moiety of the protein.

The antibody to the clostridial selenoprotein prepared by Dr. Belinda Seto failed to exhibit any cross-reactivity with either the crude or the most purified form of the M. vannielii formate dehydrogenase. Further experiments using $^{75}Se$-labeled peptide fragments from the methane bacterial protein will also be made to test for possible homology.

Keyword Descriptors:

Anaerobic electron transport and phosphorylation, selenoprotein (selenium-containing protein), glycine reductase from anaerobic bacteria, proline reductase from anaerobic bacteria, non-heme iron proteins (ferredoxins), molybdenum and tungsten in proteins, methane biosynthesis, formate dehydrogenase, acidic, heat-stable proteins, amino acid composition of selenoprotein, atomic absorption spectrometry.

Publications:

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2. J. M. Poston and T. C. Stadtman: Cobamides as Cofactors: Methylcobamides and the Synthesis of Methionine, Methane, and Acetate. In Babior, Bernard M. (Ed.): Cobalamin: Biochemistry and Pathophysiology. New York, John Wiley and Sons, Inc. 1975, pp. 111-139.

3. J. Retey, F. Kunz, T. C. Stadtman and D. Arigoni: Zur Kenntnis der β-Lysin-Mutase Reaktion: Mechanismus und sterischer Verlauf. Manuscript submitted to Helvetica.

4. F. Kunz, J. Retey, D. Arigoni, L. Tsai and T. C. Stadtman: Zur Kenntnis der β-Lysin-Mutase Reaktion: Die absolute Konfiguration der 3,5-Diaminohexansäure. Manuscript submitted to Helvetica.

Project No. Z01 HL 00206-16 LB
Laboratory of Biochemistry
Section on Intermediary
Metabolism & Bioenergetics
Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Stereochemical Studies of Enzymatic Reactions

Previous Serial Number: NHLI-11

Principal Investigator: Lin Tsai

Other Investigator: E. Caveney

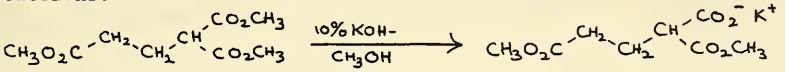
Cooperating Units: None

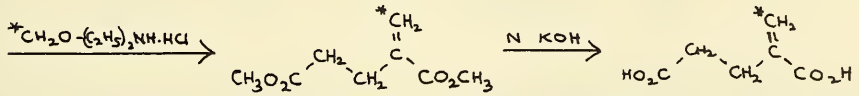
Project Description:

Objective 1: For the study of the steric course of the rearrangement catalyzed by αMG-mutase, it is necessary to establish various methodologies.

Major Findings:

1. A $^{14}C$-labelled substrate of fairly high specific radioactivity is needed as a marker for the study of the rearrangement reaction. A procedure to render optimal incorporation of $^{14}C$-atom to the methylene group of α-methyleneglutaric acid was developed according the following sequence of reactions:




After numerous experimentation, the optimum condition for the Mannich reaction was found to be the use of 50% excess of the trimethyl ester with respect to $^{14}C$-formaldehyde. Under this condition, a 55% of radioactive yield was obtained.

2. The combined enzymatic activities of αMG-mutase and MIT-isomerase were determined by a colorimetric assay of 2,4-dinitrophenylhydrazine derivative of dimethylmaleic anhydride. It was noted that the published procedure did not give consistent results; therefore, a different procedure was worked

out involving extraction of the DNP-derivative into known volume of methylene chloride, from which the chromophoric material was extracted into known volume of 2.5 N NaOH. Thus, a linear standard curve could be constructed for 0.1 - 0.4 μmole of dimethylmaleic anhydride.

3. Crude extract having αMG-mutase and MIT-isomerase activities was obtained in the 35 - 65% ammonium sulfate fraction. This usually gave a 7 - 10% conversion of α-methyleneglutarate to dimethylmaleate. Using $^{14}$C-substrate, this extract did yield $^{14}$C-dimethylmaleate of about the same specific activity, although some $^{14}$C was found in other volatile acid, probably acetic acid. The quantitative aspect of $^{14}$C-substrate to product is still under study.

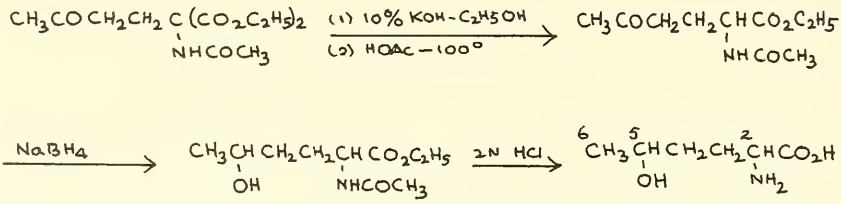
Proposed Course of Action:

To work out a procedure for degradation of dimethylmaleate to acetate so as to apply it to study the enzymatic reaction with doubly labelled substrate.

Objective 2: Since 2-amino-5-hydroxyhexanoic acid is an unusual amino acid isolated from plant, it would be of interest to determine which one of the diastereomers from the synthetic compound correspond to the natural product.

Major Findings:

An improved method for the preparation of 2-amino-5-hydroxyhexanoic acid was accomplished as outlined below:




This method gave consistently better results than the previous approach which required strongly acidic condition for the hydrolysis of the acetamidomalonate derivative. Numerous attempts were made to separate the diastereomeric mixture with only minor success. After tedious column chromatograph of the acetamidolactone derivative, only a small amount of one of the isomers could be obtained. The main difficulty in the problem is the lack of distinction between these diastereomers in their physical and chemical properties. For instance, the synthetic product, which must be a mixture of isomers, showed the same chromatographic behaviors as the natural product in TLC, amino acid analyser, as well as GLC of derivatives.

The evidence that the synthetic product was indeed a mixture of diastereomers came only after careful examination of the proton (PMR) and carbon (CMR) magnetic resonance spectra. In the PMR of the amino acid in $D_2O$, only the H at $C_2$ showed a small difference in chemical shift, 0.014 ppm. Similarly, in the CMR spectrum, the $^{13}C$ resonance of the two diastereomers differed at $C_5$ and $C_6$ by 0.10 and 0.13 ppm respectively.

Proposed Course of Action:

To continue to search for methods of separation of the isomers of synthetic product so as to correlate it with the natural product.

Keyword Descriptors:

Stereochemistry of enzyme reaction, α-methyleneglutarate mutase, coenzyme-$B_{12}$.

Honors and Awards: None

Publications: None:

Project No. Z01 HL 00207-01 LB
Laboratory of Biochemistry
Section on Intermediary
Metabolism & Bioenergetics
Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Characterization of a bacterial selenoprotein

Previous Serial Number: NHLI-15

Principal Investigator: Joyce E. Cone

Other Investigators: Thressa C. Stadtman
Raphael Martin
Belinda Seto

Cooperating Units: Joe Nathan Davis (amino acid analyses)

Project Description:

Objectives: Chemical characterization of the selenium protein required for glycine reduction in Clostridium sticklandii; purification of additional enzyme and electron transport components required for the overall reaction.

Major Findings:

The fundamental molecular properties of the selenium protein have been determined as a result of large scale purification procedures and a simplified extraction assay for glycine reductase activity. To date, studies on the identity of the selenium moiety of the protein appear to rule out selenomethionine although certain biological properties indicate the chemical form of selenium may be an ether. Although the precise nature of the selenium moiety is still unknown, sufficient amounts of protein and chemical procedures are at hand to pursue structural studies.

Proposed Course of Research:

Structural studies on the selenium moiety of protein A will be continued in conjunction with the effects of chemical modification on the biological activity of the native protein. Alternatively to its presumptive function as an electron carrier, the selenium component may serve as a group carrier during the reductive deamination of glycine to yield acetic acid.

It is also proposed to study the effects of inhibitors of protein synthesis on the production of $^{75}$Se-labeled protein in order to obtain information on whether selenium is incorporated into the protein during ribosomal protein synthesis or whether selenium is introduced into inactive (but pre-existing) protein as a "post-translational" modification.

Keyword Descriptors;

Glycine reduction, Clostridium sticklandii, selenium protein, chemical and physical characterization.

Honors and Awards: None

Publications: None

Project No. Z01 HL 00208-02 LB
1. Laboratory of Biochemistry
2. Section on Intermediary Metabolism & Bioenergetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Electron Transport System Associated with Proline Metabolism.

Previous Serial Number: NHLI-14

Principal Investigator: Belinda Seto

Other Investigators: T. C. Stadtman

Project Description:

Objectives: (1) To purify and characterize proline reductase in Clostridium sticklandii. (2) To determine the nature of the electron transfer processes involved in the reduction of proline.

Major Findings:

(1) Proline reductase has been purified to homogeneity on the basis of ultracentrifugation and gel electrophoresis. It has a molecular weight of 298,000 and consists of subunits of 61,500 and 50,500. Spectral studies indicate that it contains a flavin coenzyme. Preliminary data also suggested the incorporation of $Se^{75}$ into the protein.

(2) NADH can be used as an electron donor for crude preparations of proline reductase. However, electrons cannot be transferred directly from NADH to purified proline reductase. Presently, experiments are performed to identify the electron carrier(s) involved in proline reduction.

(3) As a cooperating project with Thressa C. Stadtman, antiserum against purified selenoprotein (protein A) of glycine reductase was prepared. The antigenic specificity of the antiserum was determined. It failed to cross react with native proline reductase or with formate dehydrogenase (Methanococcus vannielii) which is also a selenium-containing protein. The antiserum will be used specifically to study the electron transport component (selenoprotein) of glycine reductase.

Keyword Descriptors:

Proline reductase, anaerobic electron transport, flavoprotein, selenoprotein, reduced pyridine nucleotides, specific antisera.

Honors and Awards: None.

Publications: None.

Project No. Z01 HL 00209-05 LB
1. Laboratory of Biochemistry
2. Section on Intermediary Metabolism & Bioenergetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Menadione-Dependent p-nitrophenylphosphatase of Clostridium sticklandii

Previous Serial Number: NHLI-12

Principal Investigator: J. N. Davis

Other Investigator: T. C. Stadtman

Cooperating Units: None

Project Description:

Objectives: 1) To identify the natural substrate of the phosphatase. 2) To determine the composition and some physical properties of the enzyme protein.

Major Findings:

1. Titration of the reduced protein with the sulphydryl group reagent 5,5'-dithiobis (2-nitrobenzoic acid) indicated that four sulphydryl groups are present on the enzyme protein.

2. A simple and greatly improved method of isolation of the menadione dependent phosphatase in high yield was devised. This made use of the observation that the enzyme forms a tight complex with a dye known as Cibacron blue F3GA which has a high affinity for a number of enzymes involved in phosphate metabolism. When dye coupled to DEAE-cellulose was used virtually pure phosphatase could be eluted selectively in a single step procedure by the appropriate concentration of ammonium sulfate.

3. Although the fluorescense absorption and emission spectra of the pure protein indicated the presence of tryptophan, preliminary results on the amino acid composition following hydrolysis with p-toluenesulfonic acid (under conditions that do not destroy tryptophan) failed to detect this amino acid.

Proposed Course of Action:

1) Additional determinations of the amino acid composition of the phosphatase will be made (a) following carboxymethylation and hydrolysis to determine the total cysteine content and (b) following hydrolysis with methane sulfonic acid to establish the presence or absence of tryptophan.

2) Attempts will be continued to identify the natural phosphate ester substrate of the enzyme by (a) preparation of a naphthoquionol monophosphate ester and (b) by preparation of $^{32}P$-labeled extracts of C. sticklandii which can be tested as substrates. The possibility that the phosphorylated intermediate formed in the glycine reductase system may serve as substrate will be tested. The selenoprotein component of glycine reductase will be phosphorylated chemically and tested. It is of considerable interest to know whether this quinone-dependent phosphatase normally participates in a transfer process that eventually leads to ATP synthesis, whether it is involved in some energy-dependent membrane transport system that involves a quinone catalyst or is a component of a regulatory system.

Keyword Descriptors:

Menadione, quinone-dependent phosphatase, sulphydryl enzyme, p-nitrophenyl phosphatase, Clostridium sticklandii, Cibacron blue-DEAE cellulose chromatography.

Honors and Awards: None.

Publications: None.

Annual Report of the
Section on Protein Chemistry
Laboratory of Biochemistry
National Heart and Lung Institute
July 1, 1974 through June 30, 1975

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.

The metal ion and substrate binding properties of glutamine synthetase from E. coli are being studied further. A calorimetric investigation of the binding of L-glutamine to the unadenylated Mn-enzyme has given: $\Delta H^{o} \simeq -10$ kcal/mole and $K_D' \simeq 7 \times 10^{-3}$ M (in the absence of ADP) and $\Delta H^{o} \simeq -6$ kcal/mole and $K_D' \simeq 2 \times 10^{-3}$ M (in the presence of saturating ADP). Microcalorimetric measurements are being used to obtain information on protein binding sites, on the separateness of binding sites for allosteric effectors and substrates, on proton release and uptake, and on kinetic intermediates.

The ATP:glutamine synthetase adenylyltransferase, an enzyme involved in the adenylylation and deadenylylation of glutamine synthetase in E. coli, has been purified 2300-fold and partially characterized. Although this enzyme is difficult to purify, it was found that its activity could be stabilized considerably with phosphate-$MgCl_2$ buffers. Recent studies have shown that the adenylyltransferase is a single polypeptide chain of 115,000 molecular weight with $s^{o}_{20,w} = 5.6$ S. Circular dichroism measurements indicate that the enzyme has an $\alpha$-helical content of $\sim$ 26%. Amino acid analyses show 116 arginine + lysine, 248 glutamic + aspartic acids, 8 cysteine (no disulfides), 15 tryptophan, and 22 tyrosine residues per mole. The intrinsic tryptophanyl residue fluorescence of adenylyltransferase is 2-fold greater than that of free tryptophan; this property has been used to monitor ligand-induced conformational changes in the enzyme. Activators of the adenylylation reaction (ATP, L-glutamine, or the regulatory $P_{II}$ protein) produced an enhancement of fluorescence; $\alpha$-ketoglutarate, an inhibitor of adenylylation and an activator of deadenylylation, caused a net fluorescence decrease. Studies of the interaction between glutamine synthetase, adenylyltransferase, and the regulatory $P_{II}$ protein are in progress.

Studies on the glutamyl-tRNA synthetase of E. coli have indicated that this enzyme is a single polypeptide chain of $\sim$ 63,000 molecular weight. The existence of a reported complex between the catalytic unit and a regulatory protein in crude extracts could not be demonstrated. Nevertheless, bovine serum albumin or the regulatory $P_{II}$ protein activate the enzyme and decrease the $K_m'$ value for glutamate 2-fold. These effects could be related to a regulation of glutamyl-tRNA synthetase activity in the cell through a loosely associated complex between this enzyme and another protein or between this enzyme and a membrane component.

Project No. Z01 HL 00204-08 LB
Laboratory of Biochemistry
Section on Protein Chemistry
Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Protein Structure: Enzyme Action and Control

Previous Serial Number: NHLI-7

Principal Investigator: Ann Ginsburg

Other Investigators: Carlos E. Caban
Donald M. Powers
Andrew Shrake

Cooperating Units: D. Zoph, National Institute of Arthritis, Metabolism and Digestive Diseases, NIH

Project Description:

Objectives: 1) To study the physical and chemical properties of glutamine synthetase from Escherichia coli, particularly with respect to the correlation of the structure and catalytic function of this enzyme. 2) To study conformation and stabilization changes of a protein macromolecule effected through the specific binding of small molecules, and the relationship of such effects to enzyme catalysis and regulation. 3) Ultracentrifugation studies to determine macromolecular properties of biologically important proteins. 4) To purify and study the ATP-glutamine synthetase adenylyltransferase from E. coli, with emphasis on the mechanism of action and the physical structure of this enzyme. 5) To isolate the glutamyl-tRNA synthetase from E. coli in order to investigate the structure and regulation of this enzyme.

Major Findings:

1. Mutual interactions of divalent cations and other effectors with glutamine synthetase from E. coli. (Investigators: A. Shrake, D. M. Powers, and A. Ginsburg). This study is an extension of previous investigations on the role of various effectors in enzyme catalysis. A newly calibrated LKB batch-type microcalorimeter has been used to obtain the enthalpy and free energy for the binding of L-glutamine to unadenylylated glutamine synthetase in the presence of $Mn^{2+}$ at pH 7.2 (30°) from Scatchard plots of the heats of binding at different glutamine concentrations. With and without $10^{-4}$ M ADP present, respectively, $\Delta H^{o}$ $\sim$-6 and $\sim$-10 kcal/mole whereas $K_D \simeq 2$ and $\sim 7$ mM. By using two buffer systems with quite different heats of protonation in these studies, little, if any, proton uptake was found to occur upon the binding of glutamine to the native enzyme. This is in contrast to previous results obtained with freshly tightened glutamine synthetase, (i.e. enzyme from which $Mn^{2+}$ had been removed and readded). A conversion of the tightened



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form to the native conformation may occur slowly and can be investigated by calorimetry.

2. Ultracentrifugation studies. (Investigator: A. Ginsburg.) A small amount of the regulatory $P_{II}$ protein (involved in adenylylation-deadenylylation of glutamine synthetase) was purified by Dr. S. P. Adler from E. coli. Weight average molecular weights of 41,700 ± 2300 for the native protein and of 13,400 ± 2000 for the protein in 6.0 M guanidine-HCl were determined. Preliminary results indicated that there was about 8% dissociation of the tetramer to the dimer in the studies with the native protein in M/10 K-$PO_4$ buffer at pH 7.1.

Goat Anti-LND-I antibody was purified by Dr. D. Zoph (NIAMDD) to immunological and electrophoretic homogeneity by affinity chromatography, and dialyzed vs 0.02 M Na-$PO_4$ - 0.1 M NaCl buffer at pH 7.4. Sedimentation velocity studies gave $s^o_{20,w}$ = 6.76 S ± 0.02S, with no significant concentration dependence from 3.2 - 0.8 mg/ml protein concentrations. Sedimentation equilibrium studies indicated a weight average molecular weight ($M^o_w$) of 158,000 ± 6000 for this goat γ-globulin. A diffusion coefficient ($D^o_{20,w}$) of 3.8 x $10^{-7}$ $cm^2$/sec was calculated. The purified goat Anti-LNF-II antibody, in contrast to the LND-I antibody, shows some cooperativity in binding hapten which may be explored further in ultracentrifugal studies.

3. Studies on the ATP:glutamine synthetase adenylyltransferase from E. coli. (Investigators: C. E. Caban and A. Ginsburg.) Regulation of glutamine synthetase in E. coli is mediated by adenylylation and deadenylylation, a covalent modification of glutamine synthetase catalyzed by adenylyltransferase and involving also a small regulatory protein. Heretofore, purified adenylyltransferase has been unstable and consequently difficult to characterize.

A new purification procedure (with $Mg^{2+}$ included in all chromatographic steps) has resulted in a relatively stable enzyme form. In K-$PO_4$ (10 - 100 mM, pH 7.8) containing 1 mM $MgCl_2$, the enzyme was stable for months when stored at -80° or for days at 0-4° at concentrations above 1 mg/ml. At very low concentration (8 μg/ml), the half-life of the enzyme was 192 hrs at 0° or 46 hrs at 25° in this buffer. The enzyme is considerably less stable in Tris or imidazole buffers with or without added $MgCl_2$. In studies reported last year, the homogeneity of the enzyme preparation was established ($s^o_{20,w}$ = 5.6S; $M^o_w$ = 115,000 ± 5000) as was the fact that the enzyme consists of a single polypeptide chain. Circular dichroism studies indicate that the adenylyltransferase has an α-helical content of ~26% and an estimated 27% β-pleated sheet structure. Amino acid analyses show a high arginine and lysine content (116 residues/mole) and even higher levels of glutamic and aspartic acids (248 residues/mole), which are responsible for the acidic nature of the protein (pI = 4.98). In addition, the enzyme contains 8 cysteine (no disulfides), 15 tryptophan, and 22 tyrosine residues per mole. The intrinsic tryptophanyl residue fluorescence of adenylyltransferase, which is 2-fold greater than that of free tryptophan, was used to monitor ligand-induced conformational changes in the enzyme. Activators of the adenylylation

reaction (glutamine, ATP, or the regulatory $P_{II}$ protein, which itself has a very low fluorescence yield) produced an enhancement of fluorescence. A net fluorescence decrease was caused by α-ketoglutarate, which is an inhibitor of the adenylylation reaction and an activator of deadenylylation.

An analog of L-glutamine, 2-chloroketone, will be tested for a possible covalent modification of the allosteric activating site for glutamine. Determination of the stability constant and stoichiometry of the interaction between adenylyltransferase and the regulatory $P_{II}$ protein will be attempted. From previous results, we suspect that a limited proteolysis may produce an active, low molecular weight form (~70,000 mol. wt.) with a loss in its capacity to be stimulated by the regulatory protein. This and other properties of the enzyme currently are being investigated also.

4. Glutamyl-tRNA synthetase from E. coli. (Principal investigator: D. M. Powers.) Glutamyl-tRNA synthetase (GluRS) of E. coli has been reported by J. Lapointe and D. Söll (J. Biol. Chem. 247: 4966, 1972) to exist as a 102,000 MW complex consisting of a catalytic subunit (56,000 MW) and a regulatory component (46,000 MW). After finding that purification of GluRS led to a single polypeptide chain of ~63,000 mol. wt., cell extracts were examined directly for the presence of a regulatory component. For this purpose, two techniques were used: (a) The size of GluRS was determined by electrophoresis on gels containing different polyacrylamide concentrations (5-10%) by comparison with appropriate protein standards. (b) Kinetic measurements of $K_m'$ values for glutamate were made, since the presence of a regulatory component is reported to decrease this $K_m'$ value 20-fold to ~5 μM. The following strains of E. coli were examined: E. coli B, K-12, W, and K-12 (CA-244), a gift from D. Söll. Growth conditions were varied from a defined medium (glycerol or glucose + ammonia) to the enriched medium of Söll (yeast extract + tryptophan), and cells were harvested at different stages of growth. Cell extracts were prepared with a French press in a buffer of 10% glycerol, 10 mM Tris-HCl (pH 8), 10 mM $MgCl_2$, 10 mM $NH_4Cl$, and 20 mM 2-mercaptoethanol. Ribosomes and other large components were removed by high speed centrifugation or by partitioning in a two phase aqueous system. When cells were harvested at different stages of growth, GluRS activity was 1.7-fold lower in stationary than in exponential growth phase, but the $K_m'$ value for glutamate remained constant at ~50 μM. In all cases tested on polyacrylamide gels, only one peak of GluRS activity was found; this corresponded to a size of ~63,000 mol. wt. The addition of divalent cations to the cell extract ($Mg^{2+}$, $Mn^{2+}$, $Ca^{2+}$, or $Zn^{2+}$), an omission of glycerol or 2-mercaptoethanol from the cell beakage buffer, or an addition of a 0.5 M $NH_4Cl$ ribosome wash had no effect on either the GluRS molecular weight or the $K_m'$ value for glutamate.

GluRS was purified to homogeniety from E. coli B grown into exponential growth phase. A single species of 62,500 MW was observed on polyacrylamide gels in sodium dodecyl sulfate. A potential interrelationship between GluRS and the glutamine synthetase regulatory system was investigated. The adenylyltransferase from E. coli and regulatory ($P_{II}$) protein from E. coli and from P. putida were found to stimulate GluRS activity 2-fold and to decrease the $K_m'$ for glutamate 2-fold. However, bovine serum albumin at

equivalent concentrations had a similar effect; at 1 mg/ml serum albumin, GluRS activity was stimulated nearly 6-fold. Since all three proteins have approximately the same acidic isoelectric points, this effect appears to be non-specific. The effects of these acidic proteins on GluRS suggest, however, that the conformation of the enzyme may be affected by protein-protein or possibly by protein-membrane interactions. We are currently isolating sufficient quantities of GluRS to study its physical and chemical properties and to explore further the regulation of GluRS through protein-protein interactions.

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 study of structural changes that can be induced in a protein macromolecule are important in understanding cellular processes on a molecular basis.

Proposed Course of Project:

1) To study conformational and stabilization changes of a protein macromolecule effected through the specific binding of small molecules, and the relationship of such effects to enzyme catalysis and regulation. Ultracentrifugation, microcalorimetry, spectral, fluorescence, equilibrium binding, and kinetic techniques will be used. In addition, a gel method of zone transport will be standardized for measuring affinity constants of interacting molecules.

2) To study mutual interactions of divalent cations and other effectors with glutamine synthetase from E. coli.

3) Physical and chemical studies of the E. coli ATP:glutamine synthetase adenylyltransferase will be continued.

4) To purify and characterize the E. coli glutamyl-tRNA synthetase; possible mechanisms for regulating this activity in E. coli will be explored.

Keyword Descriptors:

Protein structure, enzyme catalysis and regulation, microcalorimetry, ultracentrifugation, conformation and stabilization changes in proteins, protein-protein interactions, E. coli glutamine synthetase, E. coli ATP: glutamine synthetase adenylyltransferase, E. coli glutamyl-tRNA synthetase.

Honors and Awards: None

Publications:

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3. J. B. Hunt, P. Z. Smyrniotis, A. Ginsburg, and E. R. Stadtman: Metal Ion Requirement by Glutamine Synthetase of Escherichia coli in Catalysis of $\gamma$-Glutamyl Transfer. Arch. Biochem. Biophys. 166: 102-124, 1975.

Annual Report of the
Laboratory of Cell Biology
National Heart and Lung Institute
July 1, 1974 through June 30, 1975

The Laboratory of Cell Biology was formed in November, 1974 mostly from the Sections on Cellular Physiology and Cellular Biochemistry and Ultrastructure, Laboratory of Biochemistry. Two new sections were formed: Section on Membrane Biochemistry and Section on Organelle Biochemistry. The research of the Laboratory of Cell Biology includes the biochemistry of muscle contraction; the chemistry and ultrastructure of cell motility; the structure, assembly and function of microtubules; the structural and functional interrelationships among the plasma membrane and intracellular membrane systems during endocytosis; the mechanisms of electron transport and energy trandsuction; multienzyme complexes involved in DNA synthesis; the structure and conformation of proteins.

Muscle Biochemistry: The repeating contractile unit of skeletal muscle consists of thin actin filaments attached to two Z-lines, that define the sarcomere, and thick myosin filaments that lie between the actin filaments. The cyclical interaction of the actin and myosin activates the $Mg^{++}$-ATPase activity of the myosin. In the presence of ATP, the myosin undergoes a conformational change pulling the actin filaments and attached Z-lines towards each other (contraction) as the myosin cyclically binds to and releases from the actin. During active contraction, only a small portion of the myosin molecules are attached to the actin filaments. Four other proteins, troponin I, C and T and tropomyosin are associated with the actin filaments and regulate the system by making it dependent on the presence of $Ca^{++}$, in addition to $Mg^{++}$. The detailed molecular events of this morphological contractile cycle are still incompletely understood. They cannot be studied in intact muscle or with pure actin and myosin because of the insolubility of myosin and actomyosin. In the last few years considerable progress has been made in this Laboratory by studying the model system in which myosin is replaced by its proteolytic fragments heavy meromyosin (HMM) or subfragment-1 (S-1), soluble derivatives with full enzymatic activity.

It was previously found by analytical ultracentrifugation that under conditions of excess actin and ATP, and therefore maximum ATPase activity, most of the HMM was not bound to the actin. These paradoxical data were best explained by hypothesizing a refractory state of myosin that could not bind to actin in the presence of ATP and a slow, rate-determining step for the conversion of refractory myosin to non-refractory myosin that could bind to actin in the presence of ATP.

In the last year the existence of two states of HMM were confirmed and extended to S-1 by laser-light scattering, turbidity and fluorescence measurements of the interaction of HMM and S-1 with actin in the presence and absence of ATP. It has also been shown that refractory HMM and S-1 are normal molecules, only transiently in the refractory state, by re-isolating them and showing they have normal EDTA and actin-activated $Mg^{++}$-ATPase. These experiments under steady state conditions have been supplemented by studying the interaction of actin, S-1 and ATP by measuring light scattering changes in a stop-

flow apparatus during a single catalytic cycle in which one molecule of ATP is hydrolyzed per molecule of S-1. The rate of re-binding of S-1 to actin is found to be equal to the steady state ATPase rate (measured with excess ATP), to be 10 times slower than the rate of dissociation of S-1 from actin and to become constant at high levels of actin. These data confirm the existence of a slow, rate-determining conformational change of S-1 (refractory to non-refractory state) required for its binding to actin in the presence of ATP.

When one sulfhydryl group of HMM is blocked with N-ethylmaleimide its ATPase activity is activated only 3-fold by actin, instead of the usual 200-fold. Since under conditions of maximal ATPase activity only a small portion of the NEM-HMM is bound to actin (as previously found for HMM), it was proposed that NEM-HMM also underwent a conversion from refractory to non-refractory state. This hypothesis has now been supported by demonstrating that the NEM-HMM that is not bound to actin is indistinguishable from the actin-bound NEM-HMM and that the formation of the non-refractory NEM-HMM is the rate-determining step in the hydrolysis of ATP by actin-activated NEM-HMM.

Further details of the catalytic cycle have been revealed by comparing the events occurring when only molecule of ATP is added per molecule of HMM in a stop-flow apparatus (pre-steady state kinetics) to the usual events when unlimited ATP is present (steady state kinetics). It had been found last year that ATP is bound essentially irreversibly to the active site of HMM with the rapid exponential release of 0.4 $H^+$ per bound ATP and an induced conformational change in HMM that could be measured by a change in intrinsic tryptophan fluorescence. There follows a slower exponential release of 0.6$H^+$ per ATP which equals the catalytic rate of hydrolysis under steady state conditions. The following scheme was proposed:

$$\text{myosin} + \text{ATP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{myosin-ATP} \xrightarrow{k_2} \underset{0.4H^+}{\text{myosin}^*\text{-ATP}} \xrightarrow{K_{cat}} \text{myosin} + \text{ADP} + 0.6H^+$$

The postulated two step interaction between myosin and ATP has now been supported by showing that lowering the ionic strength decreases the equilibrium constant for binding of myosin and ATP and that lowering the temperature decreases the rate of conformational change ($k_2$) and increases the association of myosin and ATP. When actin is present, ATP dissociates actin-HMM (measured by turbidity decrease) more rapidly that the induced change in fluorescence caused by ATP binding to HMM. The interaction of ATP and HMM is facilitated by the presence of actin and the rate of decay of the HMM-ATP complex (fluorescence decay) equals the rate of formation of actin-HMM (turbidity increase).

Thus, evidence has been obtained for the occurrence of molecular events in vitro that are counterparts of the mechanical events in vivo. The dissociation of actin-HMM by ATP is coincident with the formation of an HMM-ATP complex which is associated with a conformational change in the HMM protein. Hydrolysis of the ATP allows reformation of the actin-HMM complex.

Cytoplasmic Actin and Myosin: Many types of cell motility are based on cytoplasmic actins and myosins, proteins very similar to, but different from, their muscle counterparts. Several years ago we characterized these proteins in Acanthamoeba castellanii and now we are re-investigating their properties in detail. Previous efforts were hampered by the very poor yields of cytoplasmic actin from all systems. New procedures have been developed for the rapid isolation of Acanthamoeba actin in high yield and purity so that it should now be possible to characterize the protein fully.

Acanthamoeba myosin is unique among all known myosins in having a much lower molecular weight (180,000 vs about 420,000) and in its requirement for a cofactor protein for the actin-activation of its $Mg^{++}$-ATPase. Cofactor and myosin are present in only very small amounts in Acanthamoeba and their purification in quantities sufficient for the desired studies is difficult. Recent experiments indicate that cofactor and Acanthamoeba myosin can be separated from each other, and from actin, on ATP-agarose columns. A preparative procedure may be developed based on these observations.

Microfilaments and Endocytosis: One motility system thought to involve cytoplasmic actin and myosin is phagocytosis. Scanning electron micrographs show that particles to be phagocytosed by Acanthamoeba initially bind to the acanthopods (small filopodia containing bundles of actin filaments) and time-lapse motion pictures show that phagocytosis occurs within about 60 seconds of initial contact of the particle with the cell. Electron microscopy of thin-sections shows a marked accumulation of cytoplasmic actin filaments perpendicular to the limited regions of plasma membrane in contact with the particle to be ingested. As phagocytosis continues the filaments form a thick rim, lying parallel to the membrane, around the forming phagosome.There are no actin filaments associated with the internalized phagosome membrane. This rapid dissociation of filamentous actin is one of the apparent differences between cytoplasmic actin and muscle actin that needs to be studied with pure cytoplasmic actin. Within the cell the movement of phagosomes seems to be randomly controlled by cytoplasmic streaming.

Composition of Acanthamoeba plasma membrane and phagosome membrane: Previous work in this Laboratory had shown that the Acanthamoeba plasma membrane consists of about one-third each of protein, lipid (phospholipid + sterol) and a novel polymeric glycosphingolipid, lipophosphonoglycan. The proteins were shown to consist mostly of a 15,000 dalton polypeptide. Work this year has suggested that the 15,000 dalton polypeptide may be associated specifically with plasma membranes isolated from stationary phase or encysting cells and may not be a major component of the membranes of rapidly growing amoebae. Phagosome membranes are, as discussed above, derived from the plasma membrane but then fuse with the membranes of intracellular vesicles. Isolated phagosome membranes have now been found to have protein/phospholipid ratio about four times greater than the ratio for plasma membranes. This suggests that there are significant changes in the plasma membrane after it is internalized as a phagosome membrane. Dodecyl sulfate gel electrophoresis confirms the absence of actin in the phagosome membrane preparations (in contrast to its co-isolation with plasma membranes) and little, if any, of the 15,000 dalton polypeptide is present.

Membrane Fusion: During normal growth the cell surface of Acanthamoeba is internalized about 10-50 times/hour in the process of pinocytosis. Morphometric measurements of cells ingesting yeast support the hypothesis that intracellular membranes move to the surface at the same rate. The surface area of the cell remains constant during active phagocytosis but the surface area of large cytoplasmic vacuoles decreases in an amount equivalent to the plasma membrane internalized as phagosome membranes. It seems probable that the vacuole membranes lost from the cell's interior fuse with the plasma membrane. Analysis is complicated by the many fusions that occur between phagosomes and intracellular vesicles and by the probable changes in membrane proteins discussed above.

Despite the fact that the plasma membrane contains many enzymes of phospholipid metabolism, evidence has not been found for their function in the molecular events of membrane fusion. We have now shown that phospholipid bilayer vesicles fuse with the plasma membrane of viable Acanthamoeba under conditions where enzymatic catalysis is unlikely to be involved. When such fusion occurs the contents of the internal aqueous space of the phospholipid vesicle are introduced into the cytoplasm of the cell. These experiments therefore provide a basis for introducing otherwise impermeable molecules into the cell's cytoplasm. Endocytosis of phospholipid vesicles can also occur, under other conditions, as an alternate mechanism of uptake and, in this case, the vesicle and its contents are introduced into the lysosomal system of the cell.

Microtubule Assembly and Function: Cytoplasmic, flagellar and ciliary microtubules are the basis for different types of cell motility. Just as cytoplasmic microfilaments are a polymeric form of globular actin, so microtubules are cylinders consisting of 13 protofilaments each of which is formed by the polymerization of $\alpha$ and $\beta$-tubulin subunits (55,000 daltons). Polymerization and depolymerization of cytoplasmic microtubules is regulated by unknown control mechanisms. Depolymerization can be induced in vitro by low temperature, high ionic strength, colchicine or high $Ca^{++}$, none of which can reasonably be invoked for the in vivo phenomenon. Research in this Laboratory is focussed on three possible regulatory mechanisms (1) a cyclic AMP-stimulated phosphorylation of a single serine residue in $\beta$-tubulin, (2) the binding of guanine nucleotides, (3) the specific enzymatic addition of tyrosine to the COOH-terminus of $\alpha$-tubulin.

Partially purified tubulin from brain is tyrosylated by free tyrosine in the presence of ATP, $Mg^{++}$, and KCl. Highly purified tubulin is a receptor for tyrosyl groups but only in the presence of a partially purified enzyme from bovine brain. Preliminary results suggest that tubulin need not be fully tyrosylated to polymerize in vitro.

The apparent requirement for bound guanosine nucleotides for polymerization of tubulin may be more complex than previously thought. $Ca^{++}$ (1mM) inhibits tubulin polymerization but not binding of nucleotides or transphosphorylation of phosphate from free to bound nucleotides. Contrary to what was previously believed, ATP cannot be used to study the transfer of phosphate from free to bound nucleotides because ATP also phosphorylates tubulin serine residues.

GTP, however, specifically undergoes transphosphorylation and does not phosphorylate serine residues. Recent studies indicate that only half of the tubulin preparations that polymerize are substrates for the GTP-transphosphorylation reaction. It is not clear, therefore, whether, as previously supposed, phosphorylation of tubulin-bound GDP to bound GTP is a requisite for polymerization.

Flagellar microtubules are organized into a structure consisting of 9 outer doublet microtubules surrounding a central pair of single microtubules. The 9 outer doublets are connected to each other by "arms" and to the central pair by radial spokes. Sliding of the filaments is thought to be induced by an ATPase, dynein, that is a component of the arms. In addition to dynein, previous work in this Laboratory has led to the discovery of a low molecular weight Ca-ATPase in Chlamydomonas flagellae. The function of this enzyme, of dynein-ATPase and of other flagellar enzymes are being investigated. Results to date are as follows. (1) A mutant has been isolated which results in paralyzed flagellae in Chlamydomonas and in which the low molecular weight $Ca^{++}$-ATPase and the central pair of microtubules are missing. (2) Evidence has been found that certain preparations of ATP contain a specific inhibitor of dynein ATPase since with one commercial preparation of ATP the $Ca^{++}$-ATPase of dynein is normal but its $Mg^{++}$-ATPase is 90% inhibited. The nature of this inhibition is under study. (3) In addition to the dynein-ATPase and the low molecular weight $Ca^{++}$-ATPase, Chlamydomanas flagellae have been shown to contain an adenylate kinase and nucleoside diphosphokinase of unknown function in flagellar movement.

Electron Transport in E. coli and Mitochondria: Energy transduction occurs in the inner mitochondrial membrane of eukaryotic cells and in the plasma membrane of bacteria. In general terms, electrons from NADH are transferred through a series of membrane-bound intermediates to $O_2$, the terminal acceptor, which is reduced to water. Each of the intermediates has a characteristic oxidation-reduction potential (midpoint redox potential) where 50% of the molecules are reduced and 50% are oxidized. The oxidation of each intermediate can be followed by measuring the increase in absorption at a wavelength characteristic of that intermediate. By these titration curves of potential versus absorption spectra, the number of intermediates in the chain and their sequence can be determined. At several points in the passage of electrons down this electropotential gradient the energy released is converted to a form used by the cell, usually, if not always, ATP.

Data from the laboratory of Britten Chance suggested the presence in non-energized mitochondria of two forms of cytochrome b with redox potentials of -30 and +65 mV. When mitochondria were energized by ATP two forms were detected with redox potentials of +260 and +65 mV. It was suggested that the conversion of a cytochrome b from a molecule with redox potential of -30 mV to a potential of +260 mV might be the long-sought high energy intermediate.

Similar experiments in this Laboratory with E. coli membranes (non-energized) revealed three forms of cytochrome $b_1$ (redox potentials of -50, +110 and +220 mV) but none of the mid-point potentials changes when the membranes are energized by ATP. This observation led to a re-investigation of the mitochondria system. It was found that in non-energized mitochondria, in the range of +110 to +350 mV, there may be an anomalous change in absorption of cytochrome

b in a direction opposite to that to be expected. The titration data are, therefore, subject to the re-interpretation, by computer modeling, that in mitochondria, as in E. coli, there are three, not two, forms of cytochrome b. Because of the optical anomaly in non-energized mitochondria one of these may be undetected. Upon energizing the system with ATP the undetected cytochrome b may undergo a spectral shift, rather than the previously proposed change in redox potential, and is then detected. Whether this proposed ATP-induced spectral shift represents a "high-energy" intermediate remains to be determined.

DNA Synthesis in E. coli: Several lines of evidence suggest that DNA synthesis may occur in membrane-associated enzyme complexes. A search for such a system has led to the partial purification of an enzyme complex (not membrane-associated), with an apparent "molecular" weight of 390,000, that synthesizes DNA, is stimulated by ATP and prefers native to heat-denatured DNA as primer. Although the complex contains polymerase I (Kornberg's enzyme) it differs in its activity from pure polymerase I in its ATP-stimulation and its preference for native DNA. The complex also contains the recBC enzyme known to have both ATP-dependent DNase activity and DNA-dependent ATPase activity. Complexes isolated from recBC mutants that lack the DNase activity but retain the ATPase activity still show ATP-stimulated DNA synthesis in 70 mM KCl. Thus, it seems that the ATPase activity but not the DNase activity of the recBC enzyme may be required. Complexes from these mutants differ from complexes from wild-type cells, however, in being inhibited by ATP in 150 mM KCl, under which conditions the wild-type complex still shows ATP-stimulation suggesting that the apparent complex between recBC and polymerase I may be less stable in the mutant than in the wild-type cells.

Structure of Fibrinogen: Fibrinogen is the circulating protein in the blood plasma that is converted to fibrin by selective removal of a few amino acids by the specific protease thrombin. Fibrinogen (MW=335,000) consists of six polypeptide chains, two each of chains designated $\alpha$, $\beta$ and $\gamma$. Electron microscopy and thermodynamic data suggested that these polypeptides were arranged in two sets of $\alpha$, $\beta$ and $\gamma$ chains in a symmetrical molecule consisting of a central globular region (E), containing all six chains, and two identical "satellite" globular regions (D), each containing one set of three chains branching from the central region. This model has now been given strong support by quantitative kinetic analysis of the fragments produced by controlled trypsin digestion of native fibrinogen. To construct the proper model it was necessary to separate the fragments produced at different stages of proteolysis by Sephadex chromatography or sodium dodecyl sulfate electrophoresis and determine their absolute yields and molecular weights in order to account for all of the fibrinogen molecule in the products. In the earliest stages of trypsin digestion a major fragment X is formed containing the major globular regions of D and E still intact but with a loss of a number of small peptides from those portions of the six polypeptide chains that extend beyond the globular regions of D. Further digestion produces two major fragments: one D (MW=85,000) and Y (MW=134,000) which consists of a second D still linked to E. Continued proteolysis splits Y into D and E (MW=47,000) so that, finally, two D and one E are formed. This, 2D+E=170,000+47,000=217,000 with the small peptide fragments accounting for the remainder of the original mass of fibrinogen (335,000). This structure is supported by calorimetry. The thermal transitions at 61° and 100° of separated D and E are the same as those of fibrinogen indicating physical independence of the covalently-linked subunits in the native molecule.

Project No. Z01 HL 00401-09 LCB
1. Laboratory of Cell Biology
2. Membrane Enzymology
3. Bethesda, Md. 20014

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Electron transport in E. coli and rat liver mitochondria.

Previous Serial No.: NHLI-16

Principal Investigator: Richard W. Hendler

Other Investigators: None

Cooperating Units: Electrical and Electronic Engineering Section of the Biomedical Engineering and Instrumentation Branch in the Division of Research Services.

Project Description:

A. Redox potentials of b-type cytochromes of E. coli and rat liver mitochondria.

We have recently shown that E. coli possesses 3 species of cyt $b_1$ having midpoint reduction potentials about -50, +110, and +220mV. Because it has been proposed that b-type cytochromes of mammalian systems can participate in energy transduction and ATP formation, we tested whether the redox properties of any of the three was changed by the addition of ATP or the uncoupler dinitrophenol. Because reactions that phosphorylate ADP are generally sensitive to (ATP)/(ADP) (P) concentration ratios, parallel experiments were performed in phosphate-containing and phosphate-free buffers. In no case, was any evidence found for energy-dependent changes of redox potential. Having failed to confirm an energy-dependent character for redox potentials of b-type cytochromes in E. coli, we decided to study a mammalian system more closely. We found that the apparent energy-dependent change of redox potential of one of the cytochromes b in rat liver mitochondria depended on the assumption that only two species of this cytochrome were present. The data, however, were much better fit to a three component system. In this case there was no longer any evidence for the change of a low redox potential species to a high potential form. The same two species present in non-energized mitochondria (i.e. -30 and +65mV) were present in energized mitochondria. The energized mitochondria had a third species with a potential of about +260mV. The non-energized mitochondria displayed anomalous optical behavior in the voltage range where the high potential species would be revealed (i.e. +110 to +350mV). The extent of cytochrome reduction is monitored by the difference in optical absorption between a peak and a reference wavelength. This $\Delta$O.D. normally increases upon chemical reduction (i.e. a lowering of solution potential). The anomalous response

was a decrease in $\Delta$O.D. accompanying a lowering of solution potential. Therefore, instead of finding an energy-dependent change in redox potential, we observed an energy dependent optical anomaly. We believe that the phenomenon may be due to a shift in absorption spectrum so that the original peak wavelength, subsequently represents a lower optical density relative to the reference wavelength optical density. Experiments to test this explanation are further discussed below (parts C and D).

B. Determination of whether three chromatographically separated E. coli fractions containing cytochrome $b_1$, represent three different redox potential forms of cytochrome $b_1$.

We have previously separated the E. coli respiratory chain into various fractions containing different dehydrogenases and/or cytochromes. Three of these contain cytochrome $b_1$; one complexed with succinate dehydrogenase (D.E. succ), one uncomplexed (DE-Fe-2), and one associated with cytochrome oxidase (C.O.). It was found that although both "DE succ" and "DE-Fe 2" were markedly enriched with the lowest potential species, relative to the other two, all three species were present and there was no clear-cut distinction between the two fractions. "C.O." contained all three species of cyt $b_1$ with a relatively high amount of the highest potential species.

C. Redox characteristics of E. coli cytochrome oxidase (cyt d).

Cytochrome d shows a very pronounced optical anomaly in the voltage region from 50 to 200mV. Just as was seen with rat liver mitochondrial cytochrome b, the $\Delta$O.D. decreased with lower solution potentials. A voltage- (or energy-?) dependent shift in absorption spectrum could be responsible for the phenomenon. To test this idea, a series of absolute spectra were taken at different voltages. It was found that the absorption peak used for cytochrome d did shift as a function of the oxidizing potential. The nature of the shift was such that it did qualitatively account for the observed optical anomaly. A problem preventing the obtaining of accurate quantitative data is that the oxidizing potential of the cuvette-contents continually changes so that optical scans at fixed voltages cannot be obtained. In principal this kind of analysis could be applied to the energy-dependent optical anomaly of the rat liver mitochondria system. However, the spontaneous voltage drift in that system is too great. Another limitation found in the potentiometric analysis of cytochrome d was that the highest oxidizing potentials that we have been able to obtain with chemical oxidants ($K_3Fe(CN)_6$ and $KMnO_4$) are less than that normally maintained in air saturated solutions. Because of this, we have not been able to analyze an apparent very high potential component of cyt d.

D. Development of automatic and controlled voltage potentiometry.

Because of 1) The inability to obtain spectral scans at fixed voltages.
2) The inability to obtain and hold oxidation potentials above 600mV.

and 3) because of the cumbersome and laborious nature of manually performed potentiometric titrations, efforts were undertaken to improve the experimental techniques. Initial steps were directed towards developing a system using electrically controlled digital burette delivery systems to introduce chemical oxidants and reductants at rates designed to hold a fixed potential or to maintain a fixed rate of change. Recently, a radically new approach was thought of and adopted. Instead of introducing chemical oxidants and reductants, these agents are generated in situ electrically from a second set of electrodes. The generating electrode is in direct contact with the suspension of respiratory components, and the reaction products of its auxiliary electrode are isolated from the vessel by virtue of their insolubility (i.e. Ag or AgCl) and by a sleeve of KCl-AgCl-Agar. We have found that such a system can generate oxidizing potentials as high and as low as desired. Preliminary experiments have shown that fixed solution voltages can be attained and maintained as well as programmed rates of change of voltage. Problems that have not yet been resolved deal with fluctuations of voltage above and below the pre-set values due to cycling of the feed back control system and the limitations imposed by mixing times required to disperse the rapidly generated electrode products throughout the solution.

E. Proposed course.

Work will continue towards the development of the combined coulometric potentiometric system. Using this system, it should be easy to complete the potentiometric analysis for flavoproteins and cytochromes of intact E. coli, isolated components and of other respiratory systems. A new kind of overall analysis of respiratory chains may be possible. A series of complete spectral scans at fixed voltages could be used to generate a series of difference spectra as a function of voltage. These difference spectra can be mathametically resolved into individual absorption peaks which can be assigned to components of the chain based on the observed redox potentials. This approach applied to energized and non-energized respiratory chains should also reveal energy-dependent spectral shifts.

A somewhat different line of approach in this overall project will also be pursued later this year when a new visiting fellow arrives. We will try to reconstitute a functionally integrated respiratory chain from the isolated subunits we have been able to obtain.

Keywork Descriptors: Respiration, cytochromes, redox potentials, potentiometric titration, membrane function, bioenergetics, energy transduction.

Publications:

Hendler, R.W., Towne, D.W. and Shrager, R.I.: Redox properties of b-type cytochromes in Escherichia coli and rat liver mitochondria and techniques for their analysis. Biochim. Biophys. Acta. 376: 42-64 (1975).

Project No. Z01 HL 00402-03 LCB
1. Laboratory of Cell Biology
2. Section on Membrane Enzymology
3. Bethesda, Md. 20014

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: DNA synthesis in E. coli

Previous Serial No.: NHLI-17

Principal Investigators: Richard W. Hendler
Raymond Scharff
Clark Springgate

Project Description:

A. Functional differentiation between free and complexed DNA polymerase I.

At 150mM KCl, free polymerase was inhibited 44% by added ATP whereas a particulate fraction, $P_3$, was stimulated by 21%. Free polymerase was added to $P_3$ and the combined system was 27% inhibited by ATP. From the amounts of activity due to the added polymerase and that of the polymerase in $P_3$ it was calculated that the 27% inhibition was what would be expected from the arithmetic sum of 21% stimulation of the endogenous polymerase and 51% inhibition of the added enzyme. The same experiment performed at 70mM KCl showed that the free enzyme was not affected by ATP while $P_3$ was stimulated 90%. The combined system was stimulated by 34% which was the amount predicted from the summation of the two independent responses. Therefore, free DNA polymerase responds differently to ATP than does the DNA polymerase present in the cell system.

B. Ability of deoxynucleoside triphosphate (dNTP) to serve as rATP.

At 70mM KCl, ATP causes a doubling of the DNA synthesis rate. If ATP is needed for synthesis, why isn't the system more ATP-dependent? There are two obvious possibilities to explain the high background activity. One is that there may be enough endogenous non-ATP-dependent DNase activity to provide short gaps for a polymerase to copy. Further purification of the complex may then lead to a reduction of this activity. The other possibility is that dNTP, present to sustain DNA synthesis, may be used as ATP. To test this possibility, individual dNTP's or ATP was added in small aliquots to incorporating systems containing all 4 dNTP's. It was found that dATP was at least as effective as rATP, but the other dNTP's could not substitute for ATP. Therefore, even in the absence of added rATP, a background level of usable triphosphate bond energy is available to the system.

C. Effect of recBC mutation on properties of the complex.

At 150mM KCl, DNA synthesis in an extract from wild type cells was stimulated about 25% by added ATP, whereas an extract from recB$^-$ cells was inhibited by 7%. However, at 70mM KCl the mutant extract showed about 80% of the ATP stimulation of the wild type extract. Similar results were obtained with the isolated complexes from rec$^+$ and rec$^-$ cells. ATP stimulation as a function of KCl concentration showed that the mutant complex was more sensitive to salt than the wild type complex. The recBC enzyme, in addition to being an ATP-dependent DNase is also a DNA-dependent ATPase. The two activities are independent and although recBC mutants are known to be deficient in nuclease activity, their ATPase activity may be unimpaired. The recBC mutation we are studying, affected the protein so that its nuclease activity was lost and its affinity for polymerase I was altered. However, the ability to demonstrate an active complex in the mutant at low salt concentration suggests that it is not the nuclease, but rather the ATPase that is required in the complex.

The role of the ATPase has not been established, but it could be involved in unwinding the duplex in preparation for copying by polymerase.

D. Kinetics of DNA-synthesizing system.

The rate of DNA syntheses by the system under study is m kedly concentration-dependent. Time course experiments over a wide range of concentrations show that the shape of the incorporation curve goes through a continuous series of changes. The curve is sigmoidal at very low concentrations, becomes hyperbolic and then linear with increasing concentration, and starts to slope off with still higher concentrations, to produce curves with plateaus or peaks of incorporation in the middle of the incubation period. The Y-intercept obtained by extrapolation from two fixed time points (i.e. 30 and 60 mins.) is positive at very low concentrations, goes through zero and becomes negative as concentration is increased through the hyperbolic phase, increases to zero when the linear incorporation range is reached, and becomes increasingly positive as the incorporation curve slopes off. A very useful function has been developed as an indicator of the kind of incorporation curve in operation. The Y-intercept divided by the incorporation rate from 30 to 60 minutes (Y/S) allows comparisons to be made with preparations having widely different activities. The Y/S value is "+" in the sigmoidal range "0" and "-" through the hyperbolic range, "0" again at linearity, "+" during early stages of sloping off, "∞" when the 30 and 60 min. points lie on a plateau and "-" when the 60 min. incorporation point is lower than that at 30 mins. The specific activity of a given preparation increases with concentration to a maximum just before the linear incorporation rate is attained and then decreases, even to the extent of becoming negative at high concentrations. A plot of the percent of the linear incorporation rate vs. Y/S yields a curve which enables one to correct the observed specific activity at any concentration to the specific activity at linearity (i.e. Y/S=0). The kind of behavior just described is most unusual for enzymes. From some of the known characteristics of the incorporation system, however, a model has been developed which may account for the unusual

properties. The model is based on the following considerations:

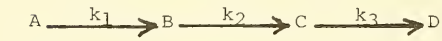
1. Duplex DNA must be "prepared" for copying by the polymerase.

2. The polymerase-recBC complex has a dissociation constant, $K_{PR}$.

3. Polymerase activity is markedly enhanced when the polymerase is complexed with recBC.

4. The complex has DNase activity associated with both of its components.

Let A represent native duplex DNA
B represent partially hydrolyzed DNA
C represent DNA containing incorporated nucleotides
D represent partially hydrolyzed DNA
$k_1$ represent rate of conversion of A to B
$k_2$ represent rate of conversion of B to C
$k_3$ represent rate of conversion of C to D




At low enzyme concentrations where much of the complex is dissociated, polymerase activity is low relative to recBC activity (i.e. $k_1/k_2$ is high). Therefore, B will tend to accumulate. As B accumulates, the rate of formation of C will increase. This can explain the lag seen at low concentrations. With increasing concentration, more complex is formed, which increases polymerase activity (decreases $k_1/k_2$) and eliminates the lag. At still higher concentrations the nuclease activity of the complex begins to prevail. During the incubation, partially digested DNA becomes more easily hydrolyzed leading to a decrease in the level of radioactive DNA. This general model has been discussed with an enzyme kineticist, John Hearon, and a mathematician experienced with kinetic problems, Richard Shrager. Both believe that it may account for the observations and are willing to collaborate in evaluating the model.

E. Dissociation and reconstitution of DNA synthesizing complex.

The ATP stimulation of DNA synthesis in crude systems is lost at 280m KCl but is completely regained by diluting the KCl concentration to 70mM. Isolated complex when re-chromatographed on Bio Gel A1.5M in 280mM KCl no longer migrates as a 390,000 molecular weight entity. Instead non-ATP stimulated polymerase activity is seen in the elution volume for DNA polymerase I, indicating the dissociation of the complex. Mixing this polymerase with a portion of the column eluate from the 270,000M.W. region (i.e. where free recBC enzyme should be located) leads to a marked enhancement of polymerase activity plus the reappearance of an ATP stimulation. The ratio of the two enzymes appears to be critical in order to achieve an ATP stimulation. Re-chromatography of the mixture on Bio Gel A1.5m, however, did not lead to the isolation of reconstituted complex. Considerations which may be pertinent to the above findings are: 1) The necessity of re-constituting

in the presence of DNA. 2) Another factor present in crude preparations may be required. 3) The two enzymes may be able to complement each other without forming a stable complex in a manner similar to that of unjoined fragments of ribonuclease.

Proposed Course of Research:

After learning of the concentration dependence of the DNA synthesizing system, it was possible to convert observed activities to uniform linear rate incorporation values. This revealed that a major part of the system was being lost to the debris fraction of the initial 20000g centrifugation. Efforts to release and retrieve this activity will be made.

The recBC enzyme will be purified and the formation of complex from pure recBC and polymerase I enzymes will be attempted. If necessary, other cell fractions will be sought to effect the formation of a stable complex. Attempts will be made to purify further the endogenous complex and to unequivocably identify recBC as a constituent. Kinetics and the concentration dependence of the purified complex will be studied. The DNA product of the purified and crude systems will be more fully characterized.

Keyword Descriptors: DNA synthesis, recombination enzymes, enzyme complex, DNA polymerase complex, recBC.

Publications:

Hendler, R.W., Pereira, M., and Scharff, R.: DNA synthesis involving a complexed form of DNA polymerase I in E. coli extracts. Proc. Nat. Acad. Sci. U.S.A. 71: (1975) (in press).

Project No. Z01 HL 00403-01 LCB
1. Laboratory of Cell Biology
2. Cellular Physiology
3. Bethesda, Md. 20014

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Differential scanning calorimetry of fibrinogen.

Principal Investigator: Elemer Mihalyi

Cooperating Units: Western Regional Research Center, Agricultural Research Service, U.S. Dept. of Agriculture, Berkeley, California 94710 Dr. John W. Donovan

Objectives: To investigate the possibility of independent thermal unfolding of subunits in the fibrinogen molecule and to correlate these subunits with the large fragments obtained by proteolysis. Further to investigate the effect of clotting on unfolding of the subunits.

Major Findings:

Solutions of fibrinogen show two endothermal (denaturing) transitions at 61° and at 100°, when heated in a differential scanning calorimeter. Similar transitions are observed for a mixture of the fragments D and E obtained by limited proteolysis of fibrinogen. Isolated fragment E shows only a single transition, at 97°. The independent thermal denaturation of these portions of fibrinogen supports the three-nodular model proposed for fibrinogen. The D and E subunits retain their characteristic denaturation behavior when fibrinogen is clotted by thrombin addition, but over a period of about one hundred clotting times, the denaturation temperature of the D subunit increases by 9° and its enthalpy of denaturation by one-third. Since this change takes place in the absence of Factor XIII activity, and its rate is proportional to thrombin concentration, it is presumed to be mediated by a proteolytic cleavage distinct from those which liberate the A and B peptide.

Methods Employed: Differential scanning calorimetry.

Project: This phase of the project completed. Further investigation will be directed toward elucidation of the mechanism of the slow action of thrombin.
Publication:

Donavan, J.W. and Mihalyi, E. Conformation of fibrinogen: Calorimetric evidence for a three-nodular structure. Proc. Nat. Aca. Sci., U.S.A. 71; 4125-4128 (1974).

Keyword Descriptors: fibrin, blood clotting, differential scanning, calorimetry, protein, subunits, thermal denaturation.

Project No. Z01 HL 00404-16 LCB
1. Laboratory of Cell Biology
2. Cellular Physiology
3. Bethesda, Md. 20014

NIH-PHS
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Proteolytic fragmentation of fibrinogen.

Principal Investigator: Elemer Mihalyi

Other Investigator: David Towne

Previous Serial No.: NHLI-153

Cooperating Units: Division of Computer Research and Technology, Laboratory of statistical and Mathematical Methodology, Richard Shrager.

Objectives: The purpose of the work performed during the last 5 years was to provide sufficiently accurate data for a complete kinetic analysis of the proteolytic degradation of fibrinogen. For this it was necessary to estimate the fraction of optical density in each of the reaction products along the reaction path. Further, the specific optical densities were needed to convert optical density distribution into mass distribution. It had to be proved that the mass distribution obeyed the law of mass conservation. The mass distribution and the independently determined molecular weights of the fragments could be used to determine the number of the fragments derived from one native molecule of fibrinogen. With all these data the kinetics could be worked out for the whole process, accounting for all the fragments formed.

Methods Employed: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis with UV-scanning, Sephadex C-200 chromatography, equilibrium ultracentrifugation, amino acid analysis, peptide mapping, kinetic modeling by the computer.

Major findings: The bovine fibrinogen-trypsin system was worked out in more detail. The optical density conversion factors of the fragments were found as follows:

Table 1

| Material | | Specific Optical Density | |
|---|---|---|---|
| Fibrinogen | 14.78 | Fragment D | 20.04 |
| Fragment X | 16.62 | Fragment E | 8.97 |
| Fragment Y | 15.61 | Fragment P1 | 21.73 |
| | | Fragment P2 | 4.60 |

With these, the sum of the calculated masses of the fragments remained constant and equal to that of native fibrinogen through the whole reaction. The mass distribution, at the stage where fragments D, E, P1 and P2 reach their



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maximum aboundance, was used to calculate the molecular weights of these components. The data are listed in table 2 together with the molecular weights determined by other methods.

The fragments were isolated by recycling on the Sephadex G200 column and were homogeneous in sodium dodecylsulfate-polyacrylamide gel electrophoresis. In high speed sedimentation equilibrium runs these gave the molecular weights listed also in table 2.

In serial digests, as judged from sodium dodecylsulfate polyacrylamide gel electrophoresis, the molecular weights of the components remained nearly constant throughout the entire digestion phase investigated here. The average of the molecular weights obtained are also given below:

Table 2

Molecular Weights Obtained by Various Methods

| Material | Sedimentation equilibrium | SDS-electrophoresis | Mass Distribution |
|---|---|---|---|
| Fibrinogen | 335,000 | ---- | ---- |
| X | 230,000 | 210,000 | ---- |
| Y | 133,800 | 132,000 | ---- |
| D | 85,000 | 85,000 | 82,300 |
| E | 47,000 | 42,000 | 51,400 |
| P1 | 16,500 | 16,000 | 17,400 |

The computer analysis showed that the peptide fractions, P1 and P2, are not connected with the fragmentation into the D and E fragments. The early phase was not resolved sufficiently in our studies, however, it is clear that the final fragment X is derived from native fibrinogen by removing all the P1 and P2. Because of this low degree of resolution the first phase, i.e. $F \rightarrow X + P1 + P2$, could be approximated as a single step and described by a single rate constant. Strictly speaking, this means that the whole P1 + P2 segment was removed in one piece. This is probably not far from truth, because other workers fround the appearance of a 40,000 molecular weight piece in the early digests and proved that this is derived from the C-terminal portion of the A$\alpha$-chains. We have also demonstrated by finger printing that P2 almost entirely originated from the same segment.

The following sequence of events: $X \rightarrow Y + D$ and $Y \rightarrow D + E$, could be described by a single rate constant, that was close to the average rate of cleavage of the peptide bonds in the slow reaction, as determined in the pH stat. However, for the curve fitting it was necessary to assume, either that there are 3-chains on either side of the D-E-D structure, that all have to be cleaved in order to separate the fragments, or that fragment X is an obligatory intermediate. The latter case would mean that P1 and P2 somehow protect the linkage between D and E, and this linkage cannot be cleaved until the protection is removed. With the protected model a single cleavage

i.e. one connecting chain, adequately describes the process. Since the chemical data show that there are 3 chains between the subunits, we prefer the first model. The data also suggest that in each chain there is only one critical bond that is cleaved. This agrees with the observation that peptide release does not seem to be associated with the fragmentation into D and E fragments. The whole analysis is remarkable for the fact that such a complicated process could be described with only two rate constants. This is the first case when a complete analysis of a proteolytic fragmentation was possible. It is axiomatic that kinetics seldom prove anything. However, in this case all the intermediates were isolated and characterized and the mechanism was suggested by the chemical data of the structure of the molecule. These facts restricted the modeling to such a degree that the results cannot be far from reality.

The data obtained for the plasmin digestion of bovine fibrinogen (NHLI-153 report 1970-1971) were not accurate for the present purposes. These experiments were redone under the improved conditions and supplemented with data on the digestion of human fibrinogen by the same enzyme. Also, the data obtained on human fibrinogen in the previous year (NHLI report 1973-74) were utilized in the computer modeling. All four systems, bovine fibrinogen and human fibrinogen cleaved by plasmin and by trypsin, were remarkably similar both with respect to fragments produced and the kinetics of the process. The main differences were with respect to the $F \rightarrow X$ step, and this is undoubtedly due to the sequence variability of the $A\alpha$-chain segment removed. The liberation of the D and E fragments followed an identical course with all four cases, only with rate differences between them. Human fibrinogen appears to be fragmented by trypsin with about half the rate observed with bovine fibrinogen. A similar rate difference, although musch less accentuated, seems to hold for the plasmin digestion of the two proteins.

Project: This phase of the project completed. Other aspects of the proteolytic fragmentation of fibrinogen will be continued.

Publications: None

Keyword Descriptors: Blood coagulation, fibrinogen, fibrinolysis, proteolytic degradation products, kinetics.

Project No. Z01 HL 00405-01 LCB
1. Laboratory of Cell Biology
2. Cellular Physiology
3. Bethesda, Md. 20014

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Circular dichroism studies on reduced alkylated lysozyme

Principal Investigator: F. H. White, Jr.

Other Investigator: A. G. Wright, Jr. (Technical)

Project Description:

Objectives:

To explore the possible appearance of conformational structure in a fully reduced, alkylated protein.

Methods:

1. Previously established methods (F.H. White, Methods in Enzymology, Vol. 25, p. 387 (1972)) were employed for the reduction of lysozyme with β-mercaptoethanol in the presence of urea, followed by alkylation with iodoacetate or iodoacetamide.

2. Methods recently developed in this lab were used for the selective alkylation of SH groups in reduced lysozyme with triphenylvinylphosphonium bromide (TVP). This reagent was originally developed by J. Swan and S.Wright Aus. J. Chem. 24, 777 (1971) for alkylation of amino groups in lysine residues.

3. Circular dichroism studies were conducted on a Cary Model 60 spectrophotometer with a Model #6001 CD attachment. The data were treated by the procedure of N. Greenfield and G. Fashman (Biochem. 8, 4108 (1969)).

4. Phosphorus assays to measure the incorporation of triphenylethylphosphonium (TEP) groups, resulting from alkylation with TVP, were conducted by the procedure of L. Lazarus and S. Chou, Anal. Biochem. 45, 557 (1972).

5. Amino acid analysis was carried out by the procedure of S. Moore and W. Stein, Anal. Biochem. 30, 1190 (1958).

Major Findings:

1. The use of TVP has been studied extensively in this laboratory and two findings have been made.

a. It has a high selectivity for SH groups between pH7 and 8.

b. Reduced TEP lysozyme is soluble up to pH6 in 0.075M sodium phosphate, whereas the carboxymethyl and carboxamidomethyl derivatives are insoluble above pH4. Hence the use of reduced TEP lysozyme made possible a study of pH effects on structure over a wider range.

2. Reduced lysozyme samples after alkylation with iodoacetate, iodoacetamide, or TVP, were examined by circular dichroism. Evidence of ordered structure was found in all samples when dissolved in either dilute phosphate or dilute HCl. The observed structures were 0-8% αhelix, approximately 30% β structure, and approximately 60% random coil.

3. Circular dichroism in 8M urea or 6M guanidine showed no evidence of structure. Digestion of reduced alkylated lysozyme samples with pepsin also destroyed the conformational structure.

Significance:

It has long been established that development of conformational structure is dependent on amino acid sequence, but the exact relationship, despite extensive empirical and theoretical studies, has never been elaborated.

The present results suggest a new approach to this problem, since it has never been firmly established that a protein chain, in the absence of disulfide bonds, could develop conformational structure to a measureable extent. Further investigation should shed some light on the amino acid sequences responsible for the various structures observed by circular dichroism.

Proposed Course:

It is proposed that this project should be continued in two directions:

a. To examine other proteins, after reduction and alkylation, for the presence of conformational structure.

b. To employ degradative procedures on reduced alkylated proteins for the purpose of identifying the smallest conformationally functional unit of amino acid sequence.

Publications:
None

Keyword Descriptors: Conformational structure, circular dichroism, reduced alkylated lysozyme, α-helix, β-structure, random coil.

Project No. Z01 HL 00406-03 LCB
1. Laboratory of cell biology
2. Cellular Physiology
3. Bethesda, Md. 20014

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Tritium labeling of binding site residues

Previous Serial No. NHLI-23

Principal Investigator: F. H. White, Jr.

Other Investigator: A. G. Wright, Jr. (Technical)

Project Description:

Objectives: To investigate the mechanism of a reaction, whereby the binding site residues of alpha-chymotrypsin become preferentially labeled with tritium.

Methods:

1. Chymotrypsin was labeled with tritiated diisopropylfluorophosphate (T-DFP) (Cohn et al., Methods in Enzymol. XI, 688 (1967)) to attach the tritiated diisopropylphosphoryl (T-DIP) group to the serine residue of Position 195. The T-DFP had been labeled by a commercial source to a specific activity of 3.3Ci/mmole, the highest available.

2. The labeled protein was exposed to tritiated hydrogen sulfide (HST) (White,et al., Radiation Res. 32, 744 (1967)), which labels the carbon free-radicals that develop from exposure to ionizing radiation.

3. Amino acid analysis with scintillation flow counting was employed for analysis of the labeled protein hydrolysate as described by F.H. White and C.R. Mencken, Anal. Biochem. 34, 470 (1968.

Major Findings:

Earlier (Ann. Rep. for 1974 (#23) and F.H. White, J. Labelled Compounds, (in press)) it was observed that the reaction of T-DIP chymotrypsin with tritiated hydrogen sulfide (HST) effected a transfer of tritium onto residues close to the binding site.

The following reactions **constitute** an hypothesis to account for this transfer, and the hypothesis was then tested as described below:

$$\text{T-DIP-chymotrypsin} \xrightarrow[\text{radiolysis}]{\text{self}} \text{T-DIP-chymotrypsin}(C^{\cdot}) \qquad (1)$$

$$\text{HST} \rightarrow \text{T-DIP-chymotrypsin (C-T)} + \text{HS}\cdot \quad (2)$$

$$\text{HS}\cdot + \text{T-DIP-chymotrypsin} \rightarrow \text{HST} + \text{DIP(C}\cdot\text{)-chymotrypsin} \quad (3)$$

A radical migration to residues close to the DIP group would be followed by:

$$\text{amino acid(C}\cdot\text{)} + \text{HST} \rightarrow \text{amino acid(C-T)} + \text{HS}\cdot \quad (4)$$

Reaction (1), whereby carbon free-radicals form on amino acid residues, ensues as a result of self-radiolysis, as shown earlier (F.H. White and G. Wright, Abstract Vth Intern. Cong. of Rad. Res., Seattle (1974)).

It is well established that HST reacts with the resulting free radical as in reaction (2) (White, et al., Radiation Res. 47, 8 (1971)), to liberate the HS· radical.

It is then hypothesized that this radical is capable of abstracting tritium from the T-DIP group, to leave a radical (C·) on the latter group, as in reaction (3). There is abundant evidence to support radical migration (e.g. see J.H. Miller, et al., Photochem. and Photobiol. 14, 577 (1971)), which would result in appearance of radicals on nearby residues. These radicals would react with HST as in reaction (4).

This hypothesis has been tested as follows, to determine whether or not abstraction of tritium by HS· proceeds under the reaction conditions employed.

Samples of tritiated lysozyme, prepared as by F. H. White et al. (Anal. Biochem. 30 295 (1969)), were exposed either to gamma-radiation or electrical discharge to create a content of carbon free-radicals approximating that produced by self-radiolysis as in reaction (1).

Subsequent exposure to $H_2S$ resulted in tritium removal from the carbon-tritium bond to a maximum of 20-30%.

Conclusions:

These results support the hypothesis that HS· abstracts tritium from the carbon-tritium bond, and therefore also support the proposed reaction mechanism.

Significance:

The tritium-labeling of binding site residues suggests applications to the study of protein binding sites. First, however, it is necessary to understand the reaction mechanism, and the present results shed light on this subject. Moreover, the abstraction of tritium from carbon by the SH radical appears not to have been demonstrated previously.



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Proposed Course of Research:

With emphasis on the possible use of this reaction in binding site studies, it is planned to continue by seeking other model protein-ligand complexes to obtain information as to the general applicability of the labeling reaction. Such information is deemed necessary prior to serious application to proteins whose structure is less well understood.

Publication:

White, F. H., Preferential tritium labelling of binding site residues in alpha chymotrypsin by exposure of the 1,3-$^{3}$H-diisopropylphosphoryl derivative to tritiated hydrogen sulfide. J. Labelled Compounds (in press).

Keyword Descriptors: Alpha-chymotrypsin, tritium-labeling, carbon free-radicals, radical migration, protein-ligand complex.

Project: Z01 HL 00407-02 LCB
1. Laboratory of Cell Biology
2. Cellular Physiology
3. Bethesda, Md. 20014

NIH-PHS
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Interaction of $SH_1$-blocked myosin with actin and ATP.

Principal Investigators: Sally Mulhern
Evan Eisenberg
W. Wayne Kielley

Project Description:
Objectives: The interaction of myosin and actin in the presence of ATP is the central reaction involved in muscle contraction. In order to understand this reaction, experiments were performed on actin and HMM which is a proteolytic digestion product of myosin. It was demonstrated that the actin can activate the HMM ATPase 200-fold while a large fraction of HMM remains not bound to actin. Since it was demonstrated in this laboratory that $SH_1$-blocked HMM (NEM-HMM) was only 3-fold activated by actin experiments were performed to find which step in the actin activation of NEM-HMM ATPase was blocked. In previous studies we demonstrated that as with unmodified HMM very little NEM-HMM is bound to actin during ATP hydrolysis under conditions of maximum actin activation. From this it was suggested that during a cycle of interaction with actin and ATP, NEM-HMM underwent a rate limiting conversion from a refractory state which is unable to bind to actin to a non-refractory state which can bind to actin. This model predicts that as with unmodified HMM the ATP turnover rate per mole of actin at high [NEM-HMM] would be much higher than the ATP turnover rate per mole of NEM-HMM at high [actin] . This conclusion depends on there being one species of modified HMM present, i.e. it must be demonstrated that NEM-HMM unbound to actin during ATP hydrolysis has the same activity as the original NEM-HMM. In the present study we determined both the ATP turnover rate per mole of NEM-HMM and actin at high [actin] and high [NEM-HMM] respectively. We also employed an analytical ultracentrifuge equipped with a separation cell to determine if the NEM-HMM which remains unbound to actin in the absence of salt and maximal actin activation has the same ATPase activity as the original NEM-HMM.

Methods Employed and Major Findings:

Double-reciprocal plots of the ATPase rate at high [NEM-HMM] in the presence of 2μM actin and double reciprocal plots of the ATPase rate at high [actin] in the presence of 5μM NEM-HMM were compared. Results demonstrated that the ATP turnover rate per mole of actin was more than 10-fold higher than the ATP turnover rate per mole of NEM-HMM both in the presence and absence

of salt. The analytical ultracentrifuge equipped with a separation cell was used to isolate the HMM which remained unbound to actin during ATP hydrolysis under conditions of nearly maximum actin activation in the presence and absence of salt. It was shown that the unbound HMM had the same 3-fold maximum actin activation just as the original NEM-HMM. These results demonstrate that NEM-HMM consists of one species which binds to actin and shows actin activation. This NEM-HMM undergoes a rate limiting transition from the refractory to the non-refractory state during interaction with actin and ATP. Since the actin activation of NEM-HMM is lower than that of normal HMM, this transition may be slower for NEM-HMM than for normal HMM.

Proposed Course of Research:

In order to determine if these findings apply to subfragment-1 which has only a single head in contrast to HMM, we propose to use the analytical ultracentrifuge to investigate the binding of NEM-subfragment-1 to actin both in the presence and absence of salt. We also plan to use the analytical ultracentrifuge to investigate the binding of actin to HMM which has been modified with NEM both at the $SH_1$ and $SH_2$ sites.

Publication:

Mulhern, S., Eisenberg, E., and Kielley, W.W. The interaction of actin with $SH_1$-blocked heavy meromyosin in the presence and absence of actin. Biochemistry (in press).

Keyword descriptors: Muscle, myosin, $SH_1$-blocked HMM, actin.

Project No. Z01 HL 00408-03 LCB
1. Laboratory of Cell Biology
2. Cellular Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Actin-myosin interaction: Control by native tropomyosin.

Previous Serial No. NHLI-26

Principal Investigator; Evan Eisenberg

Other Investigator: Louis Dobkin

Collaborating Investigators: David Kominz and Barbra Eaton, NIAMD, NIH.

Project Description:

Objectives:

It is now clear that relaxation of skeletal muscle is caused by removal of $Ca^{2+}$ from the sarcoplasm by the sarcoplasmic reticulum and that contraction is triggered by the release of the $Ca^{2+}$. It is now also clear that this effect of $Ca^{2+}$ is mediated by a complex of proteins called "native tropomyosin" which binds to the actin filament, and prevents the myosin bridges from binding to actin in the absence of $Ca^{2+}$. In previous work we investigated the activity of the three troponin components, troponin I, T and C with and without tropomyosin present. We found that all three troponin components plus tropomyosin had to be present to confer Ca-sensitivity on the interaction of actin and HMM. But we also found that troponin I and T alone could inhibit the actin-HMM interaction even without tropomyosin being present suggesting that tropomyosin may not be necessary for inhibition of the actin-HMM interaction. This result is not consistent with a recent model of troponin-tropomyosin action which suggests that tropomyosin alone blocks the binding of HMM to actin filaments with the role of the troponin being to simply orient the tropomyosin on the actin filament so as to make the blocking effect of the tropomyosin Ca-sensitive. In this model, the tropomyosin is thought to be able to occupy only two positions on the actin filamnet -- one position which blocks the actin-HMM interaction in the absence of Ca and one which accentuates it in the presence of Ca. To further investigate this question we studied the binding of tropomyosin to actin both in the presence and absence of the troponin components and correlated this binding with inhibition of the acto-HMM ATPase.

Methods Employed and Major Findings:

Direct binding studies of tropomyosin to actin using $I^{125}$ labeled tropomyosin showed that at KCl concentrations below 0.1M, tropomyosin only binds

to actin at Mg concentrations greater than 1mM. Furthermore under conditions where tropomyosin binds it always causes 60% inhibition of the actin-activated HMM ATPase. This result is not compatible with a model for tropomyosin action which allows the tropomyosin to occupy only 2 positions on the actin filament -- an on or off position. Further evidence against this simple model comes from evidence that troponin I induces tropomyosin to bind to actin under conditions where the tropomyosin itself does not bind. Since troponin I is not thought to directly interact with tropomyosin, these data suggest that troponin I may have a direct effect on the actin filament which in turn induces the tropomyosin to bind, a result which would not be compatible with the simple model of troponin-tropomyosin action given above. Further evidence for the complex interaction of these proteins comes from data showing that even under conditions where the effect of troponin I on the actin-HMM interaction is reversed by troponin C, the troponin I is still able to induce the tropomyosin to bind to actin, a result which suggests a dual role for troponin I. Finally our recent results indicate ahat although in the presence of ATP tropomyosin inhibits the actin-HMM interaction, at low ATP concentration the tropomyosin increases the HMM binding in a cooperative manner as originally suggested by A.M. Weber and her collaborators. Analogously we have found that in the absence of ATP, HMM can induce tropomyosin binding under conditions where the tropomyosin alone does not bind. However it is still not clear whether the cooperative effect of tropomyosin occurs only at low ATP concentration and also how the presence of troponin affects this cooperativity. Further work will be necessary in this area but a report of much of the above data is presently in press in Biochemistry.

Proposed Course of Research:

First the inhibition of the acto-HMM ATPase by tropomyosin alone will be investigated at high ATP concentration to determine whether the inhibition can be reversed by increasing the HMM concentration -- a result which would suggest that cooperative interaction of actin, HMM and tropomyosin occurs even at high ATP concentration. Second the activating effect which the troponin-tropomyosin complex has on the acto-HMM ATPase in the presence of Ca will be investigated to determine exactly which troponin components are necessary for this effect. We will also investigate whether this activating effect depends cooperatively on the HMM concentration both at high and low ATP concentration. Third we will continue our earlier studies in collaboration with Dr. E. Korn's group on the interaction of tropomyosin and troponin with the actin and myosin presently being isolated from Acanthameba -- an investigation which might reveal both how the troponin and tropomyosin effects depend on specific configurations of the actin and myosin and might also reveal how cellular actin differs from the actin found in organized skeletal muscle myofibrils.

Publications:

Eaton, B.L., Kominz, D.R. and Eisenberg, E. Correlation between the inhibition of the acto-heavy meromyosin ATPase and the binding of tropomyosin to F-actin: Effects of $Mg^{++}$, KCl, troponin I, and troponin C. Biochemistry

(in press).

Keyword Descriptors: Troponin, tropomyosin, muscle relaxation, actomyosin.

Serial No. Z01 HL 00409-05 LCB
1. Laboratory of Cell Biology
2. Cellular Physiology
3. Bethesda, Md. 20014

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The interaction of actin and myosin

Previous Serial No.: NHLI-25

Principal Investigators: Evan Eisenberg
W. Wayne Kielley

Collaborating Investigators for Theoretical work: Terrell Hill and Richard Podolsky, NIAMD

Collaborating Investigators for laser light scattering: Allan Fraser and Francis D. Carlson, Johns Hopkins University

Collaborating Investigators for stop flow studies: S. Chock and P.B. Chock

Other Investigators: Louis Dobkin

Project Description:

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

a study of the implication of such a state for in vivo muscle models.

Methods Employed and Major Findings:

The original finding which led us to postulate the exsitence of a refractory state is that considerable dissociation of the acto-HMM occurs even under conditions where the actin maximally activates the HMM or S-1 ATPase, as shown by ultracentrifuge and ATPase studies. We have now completed studies using laser-light scattering, turbidity and viscosity to measure the interaction of HMM and S-1 with actin. In the absence of ATP, all of these techniques suggest that marked interaction is occurring between the actin and HMM or S-1. On the other hand, in the presence of ATP,when the ATPase is nearly maximally activated by actin all three of the techniques suggest that less than 10% of the HMM or S-1 is interacting with the actin. Therefore these measurements also indicate that a large fraction of both the HMM and S-1 are in a refractory state during their interaction with actin and ATP. This work is presently in press in Biochemistry. In addition to this study we have completed a detailed analytical ultracentrifuge investigation of the binding of HMM&S-1 to actin under conditions of maximal actin-activation of the HMM and S-1 ATPase. Using a special separation cell and $ATP^{32}$ assay we have been able to isolate the HMM and S-1 which do not bind to actin, i.e., are in the refractory state, and show that they have essentially normal EDTA and actin-activated ATPase activity. Therefore, the HMM and S-1 which remain unbound to actin are the same as normal protein -- they are simply transiently in the refractory state during their interaction with actin and ATP. In the ultracentrifuge study we have also been able to demonstrate that only half as much S-1 as HMM binds to actin under conditions of maximal actin activation of the ATPase, possibly because S-1 has only one head while HMM has two heads and one bound HMM head can carry the other head down with the actin. Finally, in this ultracentrifuge study we have found that, as expected, as the KCl concentration is increased and the actin-activated ATPase decreases the amount of bound HMM at a given actin concentration also decreases. We have also been able to demonstrate that a 4-fold change in g has no effect on the amount of HMM bound, suggesting that the sedimenting process itself has no effect on the ratio of free and bound HMM. In summary the, this detailed analytical ultracentrifuge study confirms our original evidence for the refractory state. It is presently being written up for publication.

In addition to this ultracentrifuge study we have begun a stopped-flow light scattering study of the interaction of actin, S-1 and ATP. By adding a stoichiometric amount of ATP to the acto-S-1 complex, we are able to observe a single cycle of interaction of the S-1 with actin and ATP. Our results show that in this cycle, first a rapid dissociation of the acto-S-1 complex occurs, followed by a 10-fold slower rebinding of the S-1 to the actin. Furthermore, the rate of rebinding of the S-1 to actin is always equal to the steady-state ATPase rate and levels off at high actin concentration just as the steady rate does. The finding that the rate of rebinding does not increase linearly with actin concentration, but rather reaches a maximum value strongly implies that the S-1 undergoes a conformational

change prior to its rebinding to actin and at high actin concentration this conformational change becomes rate limiting. This conformational change is apparently what we have previously described as the transition from the refractory to the non-refractory state. The ability to observe a single cycle of interaction of S-1, actin and ATP should be a powerful technique for clarifying the cycle of interaction of the myosin bridge with actin myosin and ATP which occurs in vivo.

In this regard, in addition to these experimental studies, we are involved in theoretical studies on the mechanism of muscle contraction in vivo. One paper has already been published in Biophys. J. based on this work and we are presently working with Terrell Hill on a model which icnludes a refractory state as part of the mechanism of cross-bridge interaction in vivo.

Proposed Course of Research:

We plan to continue our stopped-flow experiments extending them to higher temperatures and also using HMM as well as S-1. We also plan to perform these stopped flow measurements using a 3-syringe stopped-flow apparatus which will permit us to mix the S-1 and ATP and at varying times thereafter add the actin. This will allow us to determine rate constants in the cycle which cannot be determined using the 2-syring apparatus. We also plan to continue our collaboration with Terrell Hill on the theoretical implication of the refractory state for models of muscle contraction.

Significance to Bio-Medical Research:

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

Publications:

Hill, T.L., Eisenberg, E., Chen, Y-D., and Podolsky, R.J. Some self-consistent two-state sliding filament models of muscle contraction. Biophys. J. Vol. 15: 335-372 (1975).

Fraser, A.B., Eisenberg, E., Kielley, W.W., and Carlson, F.D. The interaction of heavy meromyosin and subfragment-1 with actin: Physical measurements in the presence and absence of ATP. Biochemistry (in press).

Keyword Descriptors: Actin, myosin, muscle biochemistry, ATPase.

Project No. Z01 HL 00410-02 LCB
1. Laboratory of Cell Biology
2. Cellular Physiology
3. Bethesda, Md. 20014

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Mechanism of myosin and actomyosin-Mg-ATPase

Previous Serial No. NHLI-19

Principal Investigators: Stephen P. Chock
Evan Eisenberg

Collaborating Investigator: P. B. Chock

Project Description:

Actin and myosin appear to be ubiquitous proteins that not only provide the basis of motility in higher animals and protozoa, but also may be intimately involved in cellular development as well. In muscle, they constitute the main proteins involved in the cyclic process of myosin-actin crossbridge formation which provides the basis of muscle contraction and thus motility itself. The important roles of actin and myosin in most living systems makes it necessary for us to understand the mechanism of their function.

Objectives:

Since it is evident that muscle contraction constitutes the main aspect of animal motility, the understanding of how muscle works will no doubt provide basic information on the topic of motility as a whole. In muscle, the hydrolysis of ATP by myosin in the presence of $Mg^{++}$ provides the energy source for the contractile process. It is therefore logical to approach the problem of muscle contraction by first studying the mechanism of myosin-ATP interaction. With the information gained from the above studies one can then approach the more complex system of actin-myosin-ATP interaction which is the key event in muscle contraction itself.

In spite of the fact that the myosin and actomyosin Mg-ATPase has been studied for decades the basic information of how myosin and actomyosin interact with ATP in the presence of $Mg^{++}$ is still lacking. This information is important because it might explain how the basic biochemical steps relate to the physiological mechanism of contractile process.

Methods Employed and Major Findings:

Since steady state kinetics alone cannot provide sufficient information on the elementary steps of the interaction of myosin and actomyosin with ATP, the use of presteady state kinetics becomes necessary. With the accessibility of an excellent stopflow system designed and built by Dr. P.B. Chock here

at NIH, significant information concerning the nature of the interaction of myosin and actomyosin with ATP has been elucidated.

Pre-steady state and steady state $H^+$ release

By simultaneously monitoring both the presteady and steady state time course of $H^+$ release following the addition of ATP to heavy meromyosin (HMM, a proteolytic fragment of myosin) under conditions of excess $Mg^{++}$, 0.5M KCl pH8, 25°C, the following information concerning the interaction of myosin active site with ATP has been obtained: (1) The binding of ATP to the myosin active site is essentially irreversible and both of the HMM active sites bind ATP equally well with no observable head to head interaction during ATP binding; (2) Under the single turnover condition, that is, when the concentration of HMM active sites is less than or equal to the concentration of ATP added, the release of $H^+$ following the addition of ATP proceeds in two exponential phases with two distinct rate constants — a very fast initial phase with an apparent second order rate constant of $7X10^5 M^{-1}S^{-1}$ and a slow exponential phase with a first order rate constant, $k_{cat}$, of $0.016S^{-1}$; (3) Under steady state conditions, that is, when the concentration of ATP added is much greater than the concentration of HMM active sites, the two exponential phases of $H^+$ release are interspaced with a linear steady state. The fact that this linear steady state rate is equal to $k_{cat}$ when calculated on the basis of the molecular weight of each active HMM head, signifies that in the steady state both myosin active sites hydrolyze ATP independently and at equal rate; (4) the magnitude of the initial proton release equals $0.4H^+$ released per ATP bound, and the magnitude of the $H^+$ released in the slow exponential phase equals $0.6H^+$ released per ATP bound. Together, they total 1 $H^+$ released per ATP hydrolyzed as expected for the hydrolysis of ATP at pH8. (5) The fact that the characteristic of the initial $H^+$ burst is sufficiently different from the initial phosphate burst as reported by Taylor et al., suggests that the $H^+$ burst does not represent the phosphate burst. In other words, if Taylor's data on the rate of the phosphate burst was correct then our result suggests that the fast initial $H^+$ burst preceeds the cleavage of ATP and that no additional $H^+$ is released in the cleavage step represented by the phosphate burst until the rate limiting step controlled by $k_{cat}$. This in turn suggests that the $H^+$ burst is related to ATP binding rather than to the cleavage step as described by Taylor et al.

Pre-steady and steady state fluorescence changes

By measuring the intrinsic tryptophan fluorescence change following the addition of ATP to HMM using the stopflow technique the following events are observed: (1) There is a very fast fluorescence change following the binding of ATP with an apparent second order rate constant equal to that observed for the H+ burst; (2) under the condition of a single turnover of ATP the fast initial fluorescence change is followed by a slow exponential

fluorescence decay with a rate constant equal to $k_{cat}$; (3) Under the steady state condition, the fluorescence level remains constant for a length of time equal to the time that the steady state ATPase occurs before the ATP is depleted; (4) The amplitude of the presteady state fluorescence change is proportional to the concentration of ATP added and reaches a maximum when the concentration of ATP added equals the concentration of HMM active sites. This therefore represents a method for myosin active-site titration. Based on the above observations the following conclusions are drawn: (1) Both the preteady-state fluorescence change and the $H^+$ burst represent a conformational change in the myosin active site following the binding of ATP. (2) The $0.4H^+$ released in the burst might well represent an ionization of $H^+$ from the protein due to a conformational change following the binding of ATP. It probably does not represent the hydrolysis of ATP per se because at pH8 1 $H^+$ would be released per ATP hydrolyzed; unless the pk of bound phosphate were quite different from that of the normal phosphate. (3) Since the 2nd order rate constant for ATP binding is only $7X10^5$ which is much slower than a diffusion limited rate constant,the ATP probably binds in a 2-step process involving first the rapid formation of a collison intermediate followed by a slower conformational change. The simplest scheme that can be drawn for the interaction of myosin with ATP in the presence of $Mg^{++}$ is therefore as follows:

$$M+T \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} MT \xrightarrow[+0.4H^+]{k_2} MT^* \xrightarrow[+0.6H^+]{k_{cat}} M+Product$$

where M is myosin active site and T is ATP and MT* represents the fluorescence intermediate. The above scheme predicts that at high [T], the rate of the fluorescence burst will plateau at $k_2$. In recent work this has indeed been observed at low ionic strangth and at temperatures below 20°C.

Effect of temperature and ionic strength

The effect of temperature and ionic strength on the binding of ATP has also been studied using the fluorescence technique. The conclusions are: (1) Lowering the KCl concentration from 0.5M to 0.1M, increases the magnitude of the equilibrium constant($\frac{k_1}{k_{-1}}$) by about 6-fold, while it decreases the rate of the conformational change ($k_2$) by about 3-fold. (2) Lowering the temperature from 20°C to 10°C decreases $k_2$ by about 5-fold while it increases the value of $\frac{k_1}{k_{-1}}$ by about 3-fold. The effect of ionic strength is therefore primarily on the equilibrium step while the effect of temperature is primarily on the step involving the conformational change. This in turn suggests that the conformational change involves a large energy of activation which implies that it might involve a large cooperative structural change in the myosin following the binding of ATP.

Actomyosin-ATP interaction

By employing both light scattering and fluorescence techniques using the stopflow apparatus, the mechanism of the actin-myosin-ATP interaction has been explored. Since the association and dissociation of the actomyosin

complex can be measured by light scattering, the rate of the dissociation and reassociation of the actomyosin complex can be correlated with a decrease and increase in turbidity. In preliminary experiments we found that the rate of the turbidity decrease i.e. the rate of actomyosin dissociation in the presence of ATP, is faster than the rate of the fluorescence change; and that the maximum rate of the fluorescence change is the same whether or not actin is present. This implies that: (1) ATP dissociates actomyosin prior to the formation of the [MT*] intermediate and the conformational change occurs after the dissociation of the myosin from the actin. (2) The fact that $V_{max}$ for the formation of MT* is attained at lower ATP concentration in the presence of actin than in the absence of actin, suggests that actin enhances the accessiblity of myosin ATP-binding sites through a specific spatial orientation of the myosin head. In other words ATP binds better to actomyosin than to myosin alone. (3) The rate of decay of the [MT*] intermediate equals the rate of the turbidity rerise which in turn equals the steady state actomyosin ATPase. This suggests that not only is [MT*] an intermediate in the mechanism of the actomyosin ATPase but its disappearance is intimately involved with the rate limiting step in the cyclic interaction of myosin with actin in the presence of ATP, i.e. with the transition from the refractory to the non-refractory state. In summary, the above observations suggest that for the first time one can observe a cyclic process of actin-myosin-ATP interaction in vitro which might be analogous to the mechanistic events taking place in vivo.

Proposed course of research

More detailed experiments are still needed to clarify the elementary steps of the catalytic mechanism of both the myosin and actomyosin ATP interaction. Furthermore, it is still unclear whether the two active sites of myosin are kinetically identical. Our preliminary experiments suggest that they are the same, but more detailed studies are required before a firm conclusion can be drawn. Finally, in collaboration with Dr. Sally Mulhern we are in the process of studying the mechanism of $SH_1$-blocked myosin. This is of interest because $SH_1$-blocked myosin binds ATP almost as well as normal myosin, but its ATPase activation by actin is much decreased and we hope to determine which steps in the cycle are altered by blocking $SH_1$.

Publications:

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Keyword descriptors: Kinetics, myosin, actomyosin.

Project No. Z01 HL 00501-01 LCB
1. Laboratory of Cell Biology
2. Cellular Biochemistry and Ultrastructure
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Acanthamoeba Actin: Isolation and Role in Cell Motility

Previous Serial Number: None

Principal Investigators: Edward D. Korn
David J. Gordon

Project Description:

Objectives: In the years 1968-1972, this Laboratory provided some of the earliest and most definitive evidence for the presence of actin in cells. An isolation procedure was developed based on the known properties of skeletal muscle actin and the pure Acanthamoeba actin that was prepared was shown to be very similar, but not identical, to muscle actin in amino acid composition, polymerization to F-actin and ability to activate the $Mg^{++}$-ATPase of muscle myosin. However, the yield of actin was very low, perhaps 1% of the total cytoplasmic actin, and there were apparently significant differences noted between the Acanthamoeba and muscle actins.

This research has been re-initiated with the long range goal of understanding the role of cytoplasmic actomyosin in cell movement in Acanthamoeba castellanii and, by extrapolation, in all cells. Toward this end, the following lines of research have been initiated: 1) the isolation of pure native Acanthamoeba actin in sufficient yield for careful physical and kinetic studies. 2) Complete characterization of its physical and enzymatic properties, including viscosity, bound nucleotide, the depolymerization-polymerization cycle, binding and activation of muscle myosin subfragments, and interaction with the skeletal muscle regulatory proteins. Particular attention will be paid to those differences in properties between Acanthamoeba and muscle actin which might account for the widely differing nature of the contractile processes in these cell types. 3) Reconstruction of the Acanthamoeba actomyosin system from its components and characterization of its enzymatic properties. This system is of particular interest because of the requirement for a co-factor (as yet poorly characterized) for activation of Acanthamoeba myosin by actin. 4) Investigation of how the energy of the Acanthamoeba actomyosin ATPase system is transduced into cell movement. In particular, attention has been focused on how actin microfilaments might be anchored to the plasma membrane, with which they appear closely associated in intact cells and in isolated plasma membranes.

Methods Employed: The purification methods which have been tried individually and in combination to isolate actin from whole amoebae or from ethanol or acetone powders of amoebae include gel filtration, ion exchange chromatography, ammonium sulfate fractionation and sedimentation of actin filaments under appropriate ionic conditions. Sodium dodecyl sulfate-gels, stimulation of $Mg^{++}$ATPase activity of muscle myosin subfragments, and gross changes in viscosity, flow birefringence, and sedimentation velocity have been used to monitor the purity and state of Acanthamoeba actin preparations.

Preliminary Results: The major problems in isolating Acanthamoeba actin have been to circumvent the high proteolytic activity of the amoeba homogenate and to separate actin from myosin and other associated proteins by methods sufficiently mild to avoid denaturation. The most promising method to date involves extraction of fresh amoebae in a low salt, $Ca^{++}$-ATP buffer, adsorption of the proteins to DEAE-cellulose by a KCl-gradient. This procedure gives a high yield of 80-90%-pure actin which shows flow birefringence under polymerizing conditions and can activate muscle myosin subfragment-1 with ~15% of the specific activity of skeletal muscle actin. This activity is only slightly less than that observed by Weihing and Korn (this Laboratory, 1971) for purified actin obtained in much lower yield by an arduous procedure.

In addition to lower myosin-activation, it also appears that Acanthamoeba actin has different polymerization properties than muscle actin. In the presence of $Ca^{++}$-ATP and 0.1M KCl, muscle actin polymerizes rapidly (within seconds) at 0°C; Acanthamoeba actin remains almost totally depolymerized under these conditions, even after 36 hrs at 0°. If $Mg^{++}$ is added or if the mixture is warmed to 25°, polymerization of Acanthamoeba actin is promoted, though it is never as complete as for muscle actin. Furthermore, under conditions where muscle actin depolymerizes, some Acanthamoeba actin remains in filaments. An example of this is in preparation of plasma membranes with associated actin filaments which remain polymerized in 10mM Tris-HCl buffer. Finally, there is a tendency for Acanthamoeba actin, isolated under some conditions, to form precipitates rather than filaments when KCl is added. Further work is needed to decide which of these differences from muscle actin are trivial artifacts of the purification methods employed and which reflect physiologically important differences in the properties of cytoplasmic actin or of cytoplasmic actin in association with other proteins in the cytoplasmic contractile system.

Proposed Course of Research: Since it appears that we are close to accomplishing the first goal, the purification in high yield of Acanthamoeba actin, work next year will probably be largely concerned with fully characterizing the Acanthamoeba actin as listed in Objective 2. Should Acanthamoeba myosin and cofactor be available (see Project No. Z01 HL 00502-01 LCB) their interaction with Acanthamoeba actin will also be studied (Objective 3).

Keyword Descriptors:

Acanthamoeba castellanii, amoeba, actin, microfilaments, cytoplasmic acto-

myosin, contractile proteins, cell motility, membrane-associated proteins.

Honors and Awards: None

Publications: None

Project No. Z01 HL 00502-01 LCB
1. Laboratory of Cell Biology
2. Cellular Biochemistry and Ultrastructure
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Acanthamoeba Myosin Cofactor: Isolation and Function

Previous Serial Number: None

Principal Investigators: Edward D. Korn
Hiroshi Maruta

Project Description:

Objectives: In the years 1969-1972, this Laboratory provided some of the earliest and most definitive evidence for the presence of a myosin ATPase in the cytoplasm of cells. This molecule, together with cytoplasmic actin, is undoubtedly responsible for many types of cell motile processes in Acanthamoeba. Acanthamoeba myosin is unique among known myosins in having a much smaller molecular weight, about 180,000 vs 420,000, and in requiring a cofactor protein for actin-activation of the Acanthamoeba myosin $Mg^{++}$-ATPase. Acanthamoeba cofactor also increases the activity of muscle actomyosin. Although the Acanthamoeba myosin was previously obtained in a highly purified form, Acanthamoeba cofactor was obtained only as a relatively crude fraction separated from the myosin by hydroxylapatite, the last step in the purification of Acanthamoeba myosin. We have now renewed this research with the specific purpose of purifying the Acanthamoeba myosin cofactor and studying the nature of its interaction with Acanthamoeba actin and myosin and with skeletal muscle actin and myosin and the muscle regulatory proteins. This problem has wide implications because of the universality of cytoplasmic actin and myosin and recent evidence that some of the mammalian cytoplasmic systems may also have a cofactor protein.

Methods and Preliminary Findings:

1. Attempt to purify rapidly Acanthamoeba myosin free of actin and cofactor. This is necessary in order to have a myosin fraction suitable for the assay of cofactor activity. Crude Acanthamoeba extract can be applied to an ATP-agarose column in 0.01M imidazole, pH 7, -0.05M KCl and cofactor eluted by raising the KCl concentration to 0.1 M in the presence of 2 mM EDTA. Myosin is eluted only at 0.5M KCl. Prepared in this way, cofactor is free of myosin and actin and myosin is free of cofactor and actin. Unfortunately, the capacity of the ATP-agarose column is very low and since the cofactor and myosin each comprise less than 1% of the total protein this procedure is not suitable for large scale isolations. It may be adequate, however, for providing sufficient cofactor-free, actin-free myosin for assay purposes. Cofactor is unstable unless kept at -80° in the presence of albumin (1mg/ml).

2. Attempt to purify Acanthamoeba cofactor on DEAE-cellulose and by ammonium sulfate fractionation. The cofactor fraction from the ATP-agarose column can be adsorbed to DEAE-cellulose from 0.01M Tris-HCl, pH 8.0, -0.01M KCl and eluted with 0.045M KCl. Further purification was not possible because of rapid loss of activity. When crude Acanthamoeba extracts were applied to the DEAE-cellulose, cofactor activity was eluted only together with the myosin in 0.09-0.13M KCl and precipitated with the myosin between 1.1 and 1.6M ammonium sulfate suggesting that all of the Acanthamoeba cofactor exists as a myosin complex.

3. Attempt to purify Acanthamoeba cofactor by gel filtration. Cofactor protein and myosin partially dissociate in 0.2M KCl and cofactor elutes slightly after myosin on Sephadex G-200 and Biogel P-300 columns.

Proposed Course of Research: It is apparent that isolation of Acanthamoeba cofactor will not be easy because of its instability when separated from myosin, its low concentration in the Acanthamoeba extracts and the low capacity of the most reliable method for separating cofactor and myosin, ATP-agarose columns. Two alternatives will be pursued. First, the lengthy procedure previously developed for the purification of Acanthamoeba myosin will be carried out until the last step for which ATP-agarose separation will be substituted. In this way, by purifying the complex of Acanthamoeba myosin and cofactor, we can maintain activity of the cofactor and perhaps obtain both purified myosin and cofactor. Second, attempts will be made to purify myosin and cofactor by complexing them to polystyrene beads to which F-actin has been previously adsorbed. It has been shown by others that polystyrene will bind muscle F-actin and that muscle myosin will bind to the actin that is bound to the polystyrene. It may then be possible to recover Acanthamoeba myosin-cofactor from crude Acanthamoeba extracts or from partially purified fractions freed of actin.

Keyword Descriptors:

Acanthamoeba castellanii, amoeba, myosin, myosin cofactor, cell motility, cytoplasmic actomyosin, cytoplasmic myosin.

Honors and Awards: None

Publications: None

Project No. Z01 HL 00504-10 LCB
1. Laboratory of Cell Biology
2. Cellular Biochemistry and Ultrastructure
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Plasma Membrane and Phagosome Membranes of Acanthamoeba

Previous Serial Number: NHLI-26, NHLI-27

Principal Investigator: Edward D. Korn

Other Investigators: Sharron Smith
Shmuel Batzri

Project Description:

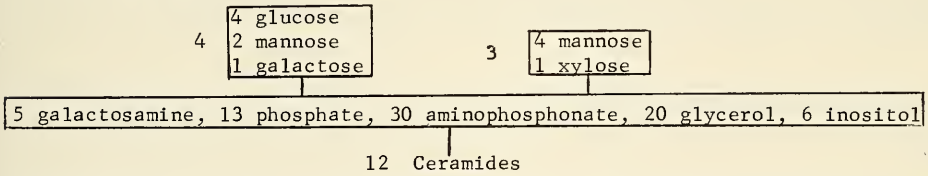
Objectives: It is the purpose of this research ultimately to determine the complete chemical composition and macromolecular organization of the amoeba plasma membrane and related intracellular membranes as well as the chemical mechanism of their fusions and interconversions. In past years we have found that the plasma membrane consists of approximately one-third each by mass of protein, lipid (phospholipids+sterols) and lipophosphonoglycan, a novel polymeric glycosphingolipid. In addition cytoplasmic actin filaments seem to be intimately associated with the plasma membrane. Phagosome membranes, which are derived from the plasma membrane but undergo subsequent fusions with intracellular membranes, have been shown to have a lipid composition similar to the plasma membrane. The plasma membrane also contains many of the enzymes of phospholipid hydrolysis and re-synthesis but is not capable of total phospholipid synthesis de novo. Work this year has largely been concerned with a more detailed comparison of the protein composition of plasma membranes and phagosome membranes and studies on the mechanism of membrane fusion with a model system of phospholipid vesicles.

Methods and Findings:
Composition of Plasma Membranes and Phagosome Membranes: Isolated plasma membranes had previously been found to contain two major polypeptides: actin and a polypeptide of about 15,000 daltons. Actin is not a true membrane component and can be removed by any of several procedures that depolymerize actin filaments. We have now found that membranes from which actin has been removed by 6M KI show a fairly constant ratio of about 0.5 mg protein/μmole of phospholipid (about 0.65 mg protein/mg phospholipid), although there may be more protein in the plasma membranes of cells at late stationary phase of growth. The 15,000 dalton polypeptide increases from a low of about 10% of the actin-free plasma membrane protein to about 50% as the cells progress from early log growth to late stationary phase. It appears likely, therefore,

that this protein is a component of the membranes of late stationary or encysting cells, some of which are present in all cultures. At all stages of cell growth the ratio of lipophosphonoglycan to phospholipid in the plasma membrane seems to be relatively constant.

Phagosomes can be readily isolated from cells that have ingested polystyrene beads and the phagosome membranes can be isolated after mild sonication. The phagosome membranes contain about the same ratio of lipophosphonoglycan/phospholipid as the plasma membranes. But data thus far collected indicate that the protein/phospholipid ratio is about 4 times higher in phagosome membranes than in plasma membranes, about 2 mg protein/μmole phospholipid. This increase in protein content is compatible with some recent freeze-cleavage electron microscopic images obtained by Dr. Blair Bowers, in this Laboratory, which show at least 10 times more intramembranous particles (presumably protein) in phagosome membranes than in the plasma membranes. Since the membranes of the vesicles with which the phagosomes fuse do not have a high frequency of intramembranous particles it seems possible that their high concentration in the phagosome membranes arises by loss of phospholipid and lipophosphonoglycan rather than acquisition of protein.

Structure of Lipophosphonoglycan: Studies on the chemical structure of this molecule have recently been resumed. The compound consists of fatty acids, neutral sugars (glucose, mannose, galactose, xylose), amino sugars, phytosphingosine, glycerol, inositol, aminophosphonic acids and phosphate. From compositional analysis of the products of partial acid and alkaline hydrolysis we have now developed the following working hypothesis for the general structure of the molecule:




Sodium dodecyl sulfate gel electrophoresis separates lipophosphonoglycan into two bands. Preliminary analysis of the two bands separated by preparative gel electrophoresis indicates that one of the bands contains xylose, mannose and a small amount of glucose but no galactose while the other band contains mannose, galactose and glucose but no xylose. These data are qualitatively in agreement with the data from the partial hydrolyses and suggest that the two postulated oligosaccharide chains may be on separable molecules.

Membrane Fusion: Studies on the interaction of single bilayer phospholipid vesicles with Acanthamoeba have been continued this year. Vesicles are made from either egg phosphatidyl choline or dipalmitoylphosphatidyl choline with the bilayer labeled with radioactive phospholipid and the internal aqueous space labeled with either radioactive glucose, radioactive inulin or amylase. Uptake by Acanthamoeba of both preparations of phospholipid vesicles is very rapid. Uptake of the egg phosphatidyl choline vesicles seems to be by endo-

cytosis since uptake is inhibited by dinitrophenol and incubation at 4° (inhibitors of endocytosis), uptake of markers of the internal aqueous space is exactly equivalent to uptake of the phospholipid bilayer and the phospholipid vesicle membranes can be visualized by electron microscopy within endocytic vesicles. Uptake of the dipalmitoyl phosphatidyl choline vesicles does not seem to be by endocytosis because it is not inhibited by dinitrophenol or 4° and occurs even when the cells are previously fixed with glutaraldehyde. Exchange of phospholipid has been ruled out by showing that the net uptake of phospholipid equals the uptake of radioactive phospholipid. Adsorption of the vesicles to the cells is eliminated by showing equal uptake of vesicles irrespective of their charge, by the difference between the dipalmitoyl phosphatidyl choline vesicles and the egg phosphatidyl choline vesicles whose surface properties are similar and by the uptake kinetics. The most likely explanation is fusion of the dipalmitoylphosphatidyl choline vesicle bilayer with the amoeba plasma membrane. This interpretation is supported by experiments with multilamellar vesicles in which it can be shown by electron microscopy that the internal vesicle membranes are introduced into the cytoplasm of the cell but are not within an endocytic vesicle. During the fusion process about 40% of the contents of unilamellar vesicles enter the Acanthamoeba. This system then provides a model for a fusion process that should allow the introduction of otherwise impermeable substances into the cytoplasm of cells. At the same time it argues that metabolic events, such as hydrolysis and re-synthesis of phospholipids, are not obligatory for membrane fusion to occur.

Proposed Course of Research:

1. The separated chains of lipophosphonoglycan will be isolated and their chemical composition determined and compared to the original material and to each other. If possible structural studies on the isolated chains will be commenced.

2. We will continue to compare the composition of the amoeba plasma membrane and phagosome membrane at different stages of growth and attempt to correlate the chemical data with the electron microscopic images obtained by Dr. Blair Bowers.

Keyword Descriptors:

Acanthamoeba castellanii, plasma membranes, phagosome membranes, membrane fusion, lipophosphonoglycan.

Honors and Awards: None

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Project No. Z01 HL 00505-09 LCB
1.Lab.Cell Biology
2.Cell.Biochem.& Ultrastructure
3.Bethesda,Marvland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Cytology of Acanthamoeba

Previous Serial Number: NHLI-28

Principal Investigator: Blair Bowers

Other Investigators: Edward D. Korn
Antoinette Ryter

Project Description:

Objectives: To elucidate the structural basis of biochemical and physiological events in the soil ameba, Acanthamoeba castellanii. Our current emphasis is on understanding more about the interrelationships and possible interconversions of internal membrane systems with those of the plasma membrane through biochemical and morphological studies of endocytosis and through morphological studies of the membranes with freeze-fracture replication and enzyme cytochemistry.

Methods Employed:
1. Transmission electron microscopy is being used for the study of fixed and embedded cells and for evaluation of isolated cell fractions.

2. The light microscope with phase contrast and Nomarski interference optics and time-lapse cinematography are being used for study of living cells.

3. The technique of freeze-fracture replication (also dependent on transmission electron microscopy) is being used for the study of surface and internal membrane morphology.

4. Routine and special biochemical and cytochemical procedures are carried out as necessary to prepare specimens for observation with the microscopic procedures and to supplement morphological data.

5. Stereological procedures are being used to quantitate and interpret electron micrographs.

Major Findings:
1. Acanthamoeba is a cell with a very high endocytic rate. This property implies a rapid turnover of surface membrane because each endocytic event carries surface membrane into the cell. It is probable that the large number of vacuoles and vesicles visible in the cytoplasm of Acanthamoeba are interrelated with the surface membrane, possibly as part of a system for recirculation of surface membrane. Although pinocytosis appears to account for the major portion of membrane turnover in cells cultured on soluble medium, a

better system for study is that of phagocytosis since the ingested particles provide convenient markers for newly internalized membranes. The problem is being approached by Dr. A. Ryter, guest worker, in two ways: one by cytochemical methods at the level of the light and electron microscopes, and the other, by using a morphometric method that determines relative surface areas of membranous structures seen in the electron micrographs.

a) The cytochemical study is attempting to localize acid phosphatase, one of the characteristic lysosomal enzymes, which is present in high amount in Acanthamoeba. The first step has been a technical study on the conditions of fixation, washing, cytochemical incubations and physiological states of the cells. All of these factors play a role in the membrane permeability to substrates used in the cytochemical localizations. In spite of many difficulties due to a low and variable permeability of the cells some quantitative results concerning the behavior of digestive vacuoles during and after phagocytosis have been obtained. Cells which have not phagocytosed particles contain on the order of sixteen vacuoles per cell. Half of these vacuoles are acid phosphatase positive (assessed from thin sections). After allowing cells to phagocytose yeast until an average uptake of 8 yeast per cell was obtained, about half of the vacuoles containing acid phosphatase disappear whereas about half of the phagosomes containing ingested yeasts become acid phosphatase positive. It is still unknown whether the apparent fusion of acid phosphatase-positive vacuoles with phagosomes occurs by direct fusion or after progressive fragmentation of the vacuoles into smaller vesicles.

b) The morphometric study is in its preliminary stages, but seems to be very fruitful. It has already been shown that during phagocytosis of yeasts, the surface area of the plasma membrane remains constant in spite of the high amount of internalized membrane. By contrast, the surface area of the internal vacuolar membranes decreases and this decrease corresponds exactly to the surface area of plasma membrane internalized as phagosomes. These observations suggest that the renewal of the plasma membrane may be made by the fusion of the vacuoles with the plasma membrane.

2. Phagocytosis is one expression of cell motility. Korn, Weihing and Pollard (in this laboratory) have previously identified and characterized the major proteins (actin, myosin and cofactor) responsible for the motility of Acanthamoeba, but the way in which these proteins function is totally unknown. Phagocytosis is an aspect of cell motility that is limited in time and space and therefore is amenable to morphological analysis. This morphological analysis may prove useful in correlating biochemical studies of motility with cell behavior. The observations in this study are relevant to our interest in plasma membrane recirculation as well. Since microfilaments (shown by Pollard and Korn to contain actin) are presumably a marker for areas of the cytoplasm that are actively participating in cellular movements, particular attention has been paid to their distribution.

Fine structural observations are necessarily of stopped events, therefore we have also made time-lapse movies of living cells to determine time periods

involved in the uptake event. In addition, observations of phagocytosing cells have been made with a scanning electron microscope (SEM) to determine if the phagocytic process is limited to certain areas of the cell and to attempt to assess the role of acanthopods in particle capture.

The movies show that contact and binding of a particle do not necessarily result in ingestion. Amebas were observed to crawl over or under particles (yeast or latex beads) or drag bound particles along with them for some time without ingestion. In one case food cup formation was observed adjacent to an adhering but non-phagocytosed particle. SEM images show clearly that acanthopods bind particles and therefore probably play an important role in particle capture. Both the movies and SEM observations show no preferential uptake by particular regions of the surface (e.g. the uropod). When the "decision" is made by the ameba to ingest, engulfment takes place rapidly and with little hesitation. Measurements of eight engulfment events from time-lapse movies shows the time elapsed between particle contact and discernible motion of the particle in cytoplasmic streaming within the cell varied between 33 and 81 seconds, most being around 60 seconds.

We have examined the process of uptake in the transmission electron microscope (TEM) with three kinds of particles: bacteria, latex beads of various sizes and lipid-extracted yeasts. The uptake of all three appear to be similar but the most informative images have been made from yeast uptake studies. The TEM images show that limited regions of membrane in contact with the particle have a marked accumulation of microfilaments associated with the membrane. The orientation of these filaments appears to be primarily perpendicular to the membrane. As the particle is enveloped by the plasma membrane the filaments may form a thick rim extending all around the particle and the predominant orientation becomes parallel to the membrane. Intermediate stages between almost complete closure of the plasma membrane around the particle and the appearance of the particle within the cytoplasm without its cortex of microfilaments are rare, presumably because this phase of engulfment is especially rapid. The channel of entry into the cytoplasm is marked for a period of time by the presence of a convoluted tubule about 45 nm in diameter that is surrounded by a compact net of microfilaments. The images suggest that the final fusion event that separates the phagosome membrane from the surface membrane is the vesiculation of a narrow tube that literally may be squeezed together by a contractile event. Once inside the cell the phagosome membrane no longer shows the association of microfilaments. The phagosome then becomes accessible to other vesicular components of the cytoplasm and a number of fusions take place. These observations point to an important role for microfilaments in the engulfment of large particles. The apparent failure of binding of a particle to the surface of the ameba to entrain ingestion and the generally capricious nature of uptake are surprising. The implication is that engulfment may be a random process that depends upon some precondition of the cytoplasm or plasma membrane.

Acanthamoeba pinocytoses continuously and one of the first fusion events of phagosomes is with pinosomes. (Pinosomes can be readily labeled with exogenous horse-radish peroxidase.) Pinocytosis appears to occur mainly by

very small vesiculations of the surface membrane and an association of microfilaments with this process has not been discerned. Subsequently hydrolytic enzymes gain access to the phagosome, but their route of entry has not yet been satisfactorily determined, since we have so far been unable to get wholly reliable cytochemical reactions for any of these enzymes.

Time-lapse cinematography shows that once the phagosome is completely severed from the plasma membrane it appears to be moved passively by cytoplasmic streaming.

3. Last year we provided a description of the structure of the plasma membrane of Acanthamoeba as shown by freeze-fracture replication. This technique reveals some aspects of the micromorphology of membranes, especially the distribution of polypeptides which are visualized as particles of various sizes and shapes. During the past year we have begun experimental manipulation of the membranes to gain insight into the nature and identity of the morphological features of the membrane.

Experiments with cationized ferritin show that both sides of the membrane are uniformly covered with negatively charged groups at least as close together as 12 nm (diameter of one ferritin molecule) and that the distribution of these charges is unrelated to the intramembranous particles. These observations confirm previous evidence that most of the charged groups on the membrane are associated with lipophosphonoglycan and provide additional information about surface charge density.

The outer surface of Acanthamoeba plasma membrane has many particles that presumably represent polypeptides extending from the cell surface. However, repeated attempts to remove the particles by exposure of intact cells to proteases in solution did not appear to alter the morphology of the outer surface of the plasma membrane. We subsequently attempted to assess the degree and specificity of digestion by monitoring the results with sodium dodecyl sulfate gel electrophoresis of isolated plasma membranes. Three enzymes were chosen for initial experiments, two broad-spectrum proteases, pronase and papain, and one more bond-specific protease, trypsin. The enzymatic digestions were carried out either on intact cells or on isolated plasma membranes. In the case of intact cells, the membranes were subsequently isolated for gel electrophoresis.

Not unexpectedly we observed that isolated membranes were much more susceptible to enzymatic attack by proteases than intact membranes. Both papain and pronase digestion of isolated membranes caused decreases in all polypeptide bands. Papain-digested intact cells showed no interpretable changes in band patterns. Trypsin digestion of either isolated membranes or intact cells was much more selective and appears to alter at least two bands. These limited results indicate that this experimental approach may be feasible but considerable further effort must be put into the basic procedures to determine why separate membrane isolations often show considerable variation in the gel pattern. Also for this project it will be desirable to find a gel system that provides higher resolution of the membrane polypeptides.

Our interpretation of this data, taken together with freeze-fracture observations, is that the outer surface of Acanthamoeba probably does contain exposed polypeptides, but the failure of some proteases to attack intact cells is probably due to some protective function of the lipophosphonoglycan.

Significance to Bio-medical Research: Phagocytosis is a major mechanism of defense against infection and pinocytosis promises a means of therapy in certain storage diseases in which lysosomal enzymes are defective. Both of these processes take place at high rates in the ameba and can easily be studied in Acanthamoeba.

Changes in cell surface properties appear to play a major role in proliferation of cancerous cells. Acanthamoeba is in some senses an analog of transformed cells and thus information on the organization of its surface membrane may prove useful in understanding malignant cells.

Proposed Course of Research: The interrelationships of surface membrane and internal membrane systems in Acanthamoeba castellanii will be studied by cytochemical and stereological techniques and with freeze-fracture replication.

Keyword Descriptors:

Membrane morphology, endocytosis, Acanthamoeba castellanii, electron microscopic, freeze-fracture, plasma membrane

Honors and Awards: None

Publications:

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Project No. Z01 HL 00503-03 LCB
1. Laboratory of Cell Biology
2. Section on Organelle Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Structure, Assembly and Function of Microtubules

Previous Serial Number: NHLI-6

Principal Investigators: Martin Flavin
Yoko Nagata
Daniel Raybin
Keith Summers

Project Description:

Objectives: Microtubules, found in the cytoplasm and dividing nuclei of all eukaryotic cells, are cylinders composed of 13 parallel protofilaments, formed by the polymerization of a dimeric precursor consisting of 2 very similar 55,000 dalton subunits, $\alpha$ and $\beta$ tubulin. Polymerization is induced by warming suitable brain extracts, and we measure it by light scattering, low-speed centrifugation of polymer, electron microscopy or, recently, by darkfield light microscopy. Polymerization is reversed by cooling and tubulin can be purified by repeated cycles of warming and cooling. This in vitro polymerization appears to be nucleated by open rings of short protofilaments which grow into sheets prior to closure to cylinders. Whether this sequence precisely matches in vivo assembly is not known. Polymers consisting only of tubulin have been reported to form slowly under special circumstances, but usually small amounts of certain other proteins copolymerize. The most conspicuous of these MAPS (microtubule associated proteins) bands as 2 high molecular weight proteins in sodium dodecyl sulfate gel electrophoresis, appears to form a filamentous coating on the outer microtubule surface, and may be related to flagellar dynein. The status of other MAPS is still obscure, but they probably include a protein kinase and a tubulin-GDP transphosphorylase.

Microtubules make up the mitotic spindle responsible for chromosome movements, and are essential for intracellular transport of material and for flagellar and ciliary motility. They are also implicated in the development and maintenance of anisometric cell form, and thus may lead towards a molecular understanding of cellular morphogenesis. In living cells microtubules may form and dissolve quite rapidly, for example in the mitotic spindle and in relation to the movement of pigment granules, at a time when the total cell content of tubulin appears constant. Although all tubulins studied appear to be structurally very similar, it is possible in some cases that there may be multiple tubulin genes coding proteins destined only for one or another specific organelle. More generally it seems that transcriptional and translational

controls could not be sufficient to regulate microtubule assembly in vivo. Moreover, none of the conditions currently used to depolymerize microtubules in vitro (cold, high ionic strength, colchicine, mM $Ca^{+2}$) is likely to have a regulatory function in vivo. Our research is now focussed on certain post-translation modifications of tubulin which might modulate the assembly, or functions, of microtubules. One of these is a cAMP stimulated phosphorylation of a single serine residue in β-tubulin (several residues in a high molecular weight MAPS are also phosphorylated). Second, tubulin dimer binds guanine nucleotides at 2 sites, one exchangeable (E) and one not (N), and some evidence has suggested that polymerization involves the transformations indicated by:

$$\text{E-dimer-N-GDP} \xrightarrow{\text{GTP}} \text{GTP-E-dimer-N-GDP} \xrightarrow{\text{ATP}} \text{GTP-E-dimer-N-GTP} \twoheadrightarrow \text{GDP-E-polymer-N-GDP}$$

(or GTP)

Although an enzyme catalyzing transphosphorylation of the N-GDP has not been identified, and there is conflicting evidence as to whether the polymer contains only GDP, nucleotide binding and transphosphorylation constitute tubulin modifications which might control assembly or function. A third post-translational modification which we have recently identified is the specific addition of a tyrosine residue to α-tubulin, apparently by peptide linkage at the C-terminal end of the polypeptide chain.

Flagellar microtubules consist of an array of 9 parallel outer doublet microtubules surrounding a central pair of single microtubules. Paired arms extend from one tubule of each outer doublet towards the adjacent doublet, and radial spokes extend inward from each doublet, terminating in enlarged spoke heads at a central sheath which surrounds the central microtubules. ATP is utilized for motility by energy-transducing protein(s) called dynein, which are located in the outer doublet arms, and are believed to induce sliding of doublets past each other by translocation through binding sites on the adjacent doublet. Dynein can be solubilized as a heterogeneous, high molecular weight ATPase, activated by either $Mg^{+2}$ or $Ca^{+2}$. We have previously reported that Chlamydomonas flagella contain, in addition to dynein, a distinct low molecular weight, $Ca^{+2}$-specific ATPase. Our objective is to determine the function of this enzyme, and we consider two principal possibilities. There is evidence that during flagellar bending there must be translocation of spoke heads along the central sheath, and the enzyme might be derived from a spoke head energy-transducing system. Secondly, the enzyme might function in steering or tactic responses, in which $Ca^{+2}$, and possibly ATP as well, have been implicated.

Major Findings:

1. Post-translational modifications of tubulin and microtubule assembly.

1a. Tyrosylation of α-tubulin (D. Raybin): It was recently shown that rat brain extracts could incorporate a tyrosine residue into a specific protein having many of the characteristics of tubulin. The incorporation appeared to be through a peptide bond at the C-terminal end of the protein. The reaction utilized free tyrosine rather than tyrosyl-tRNA, required only ATP, $Mg^{+2}$ and KCl, and appears to be unprecedented. We have confirmed these results and

shown by high resolution SDS gel electrophoresis that α-tubulin is the only protein in brain extracts which incorporates radioactive tyrosine. Tubulin purified by ion-exchange chromatography retains the over-all reaction, but tubulin purified by 3 cycles of polymerization does not. Using the latter as substrate, we have been able to establish an assay for the tyrosylating enzyme, and to partially purify it from bovine brain and separate it from tubulin. Tubulin purified by 3 cycles of polymerization has the capacity to accept at least 0.25 moles of tyrosine per mole of α-tubulin. At the same time, the radioactivity incorporated by a crude extract can copolymerize through several cycles with added purified tubulin. These preliminary results suggest at least that tubulin can polymerize in vitro without being exclusively in the tyrosylated or detyrosylated state.

1b. Tubulin bound nucleotides (Y. Nagata, D. Raybin): The assay for GTP binding to the exchangeable (E) site and for transphosphorylation of GDP at the non-exchangeable (N) site is based on incubating tubulin in the presence of colchicine with $H^3$, $\gamma$-$P^{32}$-labeled GTP or ATP. Free nucleotides are rapidly separated from tubulin dimer; the $H^3$ in the latter indicates the extent of binding at the E site, and the excess of $P^{32}$ over $H^3$ indicates the extent of transphosphorylation. Since the assay is based on isotope ratios, we first ascertained that tubulin did not form any guanosine polyphosphates; specifically no ppGpp (or cGMP) was formed even in the presence of trapping pools. Concentrations of $Ca^{+2}$ which inhibit polymerization (1 mM) did not inhibit binding or transphosphorylation. A closer examination of the inhibition of polymerization by $Ca^{+2}$ was also consistent with this result: by varying the concentration of free $Ca^{+2}$ in the presence of 2 fixed concentrations of Ca-GTP chelate it was shown that free $Ca^{+2}$ was the active inhibitory species. Contrary to previous results we found that ATP could not be used to study transphosphorylation: instead of a maximum of 1 mole of "excess" $P^{32}$ bound per tubulin dimer, 2 or, in the presence of cAMP, 3 moles were bound, much of which was covalently linked, presumably to serine residues. This phenomenon was not observed with GTP. With GTP a high extent (0.5 - 1.0 moles excess $P^{32}$/mole tubulin) of transphosphorylation has been observed with only half of our tubulin preparations which are competent to polymerize; either transphosphorylation is not prerequisite to polymerization or the tubulin is already charged with GTP on the N site and cannot accept additional $P^{32}$. However the tubulin was obtained by cold-depolymerization after 3 cycles of polymerization and, according to the results of others, should contain only GDP. Therefore we are now trying to identify directly the nucleotides bound in the polymer; the problem is to remove unbound nucleotides completely without causing depolymerization.

1c. Direct observation of microtubule polymerization and depolymerization by darkfield light microscopy (K. Summers and D. Raybin). Previous electron microscopic observations of polymerization have indicated that some form of nucleation may be required and that open sheets of protofilaments are intermediates. Drawbacks are that observation can only be made at arbitrary intervals and artifacts may be produced by fixation and drying the material on grids for negative staining. Although the diameter of a microtubule (25 nanometers) is only 1/10 of the resolving power of the light microscope, we

have found that they can be clearly visualized by darkfield illumination. Microtubules can be observed directly and continuously under in vitro polymerization and depolymerization conditions. Length distributions can easily be determined, and we find that completed microtubules maintain a straight configuration even after achieving a length 1000 times their diameter. Intermediate stages, which may correspond to long open sheets, are more flexible and less stable. Depolymerization by $Ca^{+2}$ seems to involve destabilization along the entire length of the microtubule, whereas colchicine induces depolymerization from one end. A curling up is sometimes observed in the absence of GTP.

2. Flagellar Motility.

2a. The function of the low molecular weight $Ca^{+2}$-activated ATPase in Chlamydomonas flagella (K. Summers). The localization of this enzyme in the flagella should give a clue to its function, and one approach to localization is to see if it is altered in quality or quantity in mutants with specific flagellar structural defects. Four genes have been identified mutations in which result in paralyzed flagella lacking the central pair of microtubules. We have examined 3 of these and found that the Ca-ATPase is similar to that of wild type in 2, but is probably totally absent from the third, pf-15. Further work is required to rule out the possibility that this result might be due to increased leakiness or fragility of the pf-15 flagella.

2b. Specific inhibitors or activators of dynein (Y. Nagata and T. Watanabe). Solubilized dynein has an ATPase activity activated almost equally well by $Ca^{+2}$ or $Mg^{+2}$. We have one commercial preparation of ATP with which the $Ca^{+2}$-ATPase activity is normal, but the $Mg^{+2}$-ATPase is reduced by 90%. Either traces of an inhibitor are present in it, or traces of an activator in all other preparations. The inhibitory preparation has lower amounts of many metals including calcium; $Ca^{+2}$ supplementation does not reverse the inhibition. It has a higher concentration of iron.

2c. Flagellar adenylate kinase and nucleoside diphosphokinase (T. Watanabe). Last year's report described the properties of these enzymes and their possible significance for flagellar regeneration and motility. Although not currently being pursued, further work showed the presence in Chlamydomonas of at least 3 molecular species of each enzyme. For each enzyme one species appeared to be uniquely flagellar and one to be shared with cell bodies. Another project of last year's report not currently being pursued is the study of mutants resistant to drugs which inhibit microtubule assembly or function.

Proposed Course of Research: We plan to purify the tubulin tyrosylating enzyme, to characterize the reaction, and determine whether tyrosine removal is catalyzed by the same enzyme. The reaction seems quite specific for tyrosine (only phenylalanine has been shown to replace it, with a $K_m$ 100 times higher); however it is possible that free tyrosine is not the actual substrate. For example the enzyme might function in the incorporation of tubulin into membranes, and it may be informative to determine the cellular localization of the enzyme. We will determine whether flagella contain the enzyme and

whether flagellar doublet microtubules can be tyrosylated. We hope to obtain $\alpha$-tubulin in completely tyrosylated and detyrosylated states, and then to assess the role of this modification in the conversion of monomer to dimer, dimer to polymer, or in microtubule function. If both forms can polymerize in vitro we will analyze the polymers by microscopy, compare the kinetics of polymerization, and determine which MAPS copolymerize by gel electrophoresis. Eventually we will examine cells in which microtubules undergo rapid reversible assembly to see whether these changes correlate with extent of tyrosylation.

With regard to tubulin nucleotides, we will try various procedures to determine what nucleotides are present in the polymer and whether hydrolysis of both GTPs is essential for polymerization. We will determine whether the transphosphorylation reaction is catalyzed by tubulin itself or by a separable enzyme. In the latter case we will purify the enzyme in the expectation that it is likely to be a regulatory protein.

If we confirm that the absence of the Ca-ATPase from pf-15 flagella reflects its association with central structures of the flagella, we will characterize these mutant flagella further by electron microscopy and SDS gel electrophoresis. We may be interested to determine enzyme patterns in phototaxis as well as other paralyzed mutants, and in other species; we know that the Ca-ATPase is absent from extracts of Tetrahymena cilia, but a wider survey is needed to ascertain whether there is a significant correlation with the different motility systems of ciliates, biflagellates and monoflagellates. Apart from the Ca-ATPase, we are interested in the broader problem of the involvement of $Ca^{+2}$ in cell steering. For this purpose we would like to work with reactivated flagella, i.e. detached flagella which have been partially demembranated and become motile only when ATP is added, and to see at first whether $Ca^{+2}$ affects the asymetry of flagellar beating.

Keyword Descriptors:

Cilia, microtubules, motility

Honors and Awards: None.

Publications:

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ANNUAL REPORT OF THE
LABORATORY OF CELLULAR METABOLISM
NATIONAL HEART AND LUNG INSTITUTE
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Although there have been some changes in emphasis as well as in the direction and scope of specific projects during the past year, the research program of the Laboratory of Cellular Metabolism continues to be concentrated in five main areas. These are (1) the control of cyclic nucleotide phosphodiesterase and adenylate cyclase activities, (2) the metabolism and functions of cyclic nucleotides in lung, leukocytes and arterial smooth muscle, (3) the regulation of hormone-sensitive lipase activity in adipose tissue, (4) the mechanisms by which histamine release from mast cells is initiated and terminated, (5) the control of lipid synthesis, particularly cholesterol synthesis in mammalian cells. Some of the major findings are outlined below.

1. Control of Cyclic Nucleotide Phosphodiesterase and Adenylate Cyclase Activities.

We have had a long standing interest in the mechanisms by which cyclic nucleotide degradation is regulated and were among the first to show that cyclic AMP phosphodiesterase activity can be (1) rapidly increased in fat cells by insulin and cyclic AMP, (2) "induced" in cultured fibroblasts by maintaining intracellular cyclic AMP at an elevated level for many hours and (3) decreased in hepatoma cells by glucocorticoids. We have reported that in fat cells it is a particulate high affinity phosphodiesterase whose activity is altered by insulin, dexamethasone and cyclic AMP. Numerous attempts over the past two years to solubilize this enzyme (or enzymes) for further study have met with little success. Recently, however, we have discovered a method for reproducibly solubilizing the membrane-bound phosphodiesterase activity in essentially 100% yield and hope that purification by standard techniques of ion exchange, affinity and gel chromatography will now be possible.

Another phosphodiesterase with particularly interesting regulatory properties has been partially purified from the soluble fraction of rat liver. The kinetics of cyclic AMP hydrolysis with this enzyme are consistent with those of an allosteric enzyme displaying positive cooperativity between catalytic sites. We have now completed detailed kinetic studies of the effects of (1) cyclic GMP, cyclic IMP and cyclic XMP on cyclic AMP hydrolysis, (2) cyclic IMP, cyclic AMP and cyclic XMP on cyclic GMP hydrolysis, and (3) cyclic GMP, cyclic AMP and cyclic XMP on cyclic IMP hydrolysis. It appears that this phosphodiesterase favors cyclic GMP both as a substrate and as an effector. We are at present working to complete purification of the enzyme as a prerequisite to characterization of its structural and regulatory properties.

Work on adenylate cyclases this year has been relatively more limited than in the recent past. The effects of choleragen on the enzyme in rat

fat cells and in cultured fibroblasts have been investigated. The latter cells seem likely to be especially useful for study of the nature of the choleragen receptor. The fibroblasts lack the capacity to synthesize $GM_1$ ganglioside which has been postulated by several workers to be the receptor or a critical part of it. When grown in the usual manner, however, they take up and incorporate gangliosides from the serum contained in the medium. Using a serum-free medium we have now grown cells that are deficient in $GM_1$ ganglioside and in which the adenylate cyclase does not respond to choleragen. By restoration of the ganglioside in current experiments, we expect to learn more about the nature of the choleragen receptor and its relation to adenylate cyclase.

2. Metabolism of Cyclic Nucleotides in Lung, Leukocytes and Arterial Smooth Muscle.

When these studies were initiated several years ago, virtually nothing was known about the factors that influence cyclic GMP metabolism or the functions subserved by this nucleotide in mammalian cells. Our experiments with lung slices provided evidence that bradykinin as well as acetylcholine (which was shown elsewhere to act in other tissues) could cause accumulation of cyclic GMP and led us to suggest that cyclic GMP might play a role in the regulation of prostaglandin synthesis. In recent work we have used two other systems that provide relatively more homogeneous populations of cells and a better opportunity for correlation of changes in cyclic nucleotide content with modifications of cellular function.

The availability of a large body of physiological data on the human umbilical artery suggested that it would be useful for study as an example of vascular smooth muscle. This proved to be the case. It was found that acetylcholine, bradykinin, histamine, serotonin and $K^+$ ions, all of which produce contraction of the artery, also cause a rapid accumulation of cyclic GMP in this tissue. These agents do not alter the cyclic AMP content of the artery which we have found to be elevated only be prostaglandin $E_1$ ($PGE_1$), an agonist that induces relaxation of the artery. In many tissues calcium ($Ca^{++}$) plays a critical role in cyclic nucleotide metabolism. The effects of acetylcholine, bradykinin, histamine and $K^+$ were abolished in the $Ca^{++}$-depleted artery and restored after return of $Ca^{++}$ to the incubation medium. Two ionophores (drugs that facilitate the movement of $Ca^{++}$ through membranes) mimicked the effects of these $Ca^{++}$-dependent agonists on cyclic GMP. Accumulation of cyclic GMP induced by serotonin, on the other hand, was not diminished in $Ca^{++}$-depleted arteries and, in fact, seemed to be inhibited by $Ca^{++}$ at the concentration usually present in the medium. These studies demonstrated for the first time the existence of two different mechanisms for control of cyclic GMP accumulation. One, $Ca^{++}$-dependent, has been observed in several other tissues. The other, not requiring exogenous $Ca^{++}$ has thus far, to our knowledge, been found only in the umbilical artery and in human leukocytes (see below).

Oxygen ($O_2$) acts in two (or more) separate ways to initiate closure of the umbilical artery at birth. It, apparently directly, causes contraction and further plays a "permissive" role in the action of other chemical agents that cause contraction. We have found that $O_2$ rapidly raises the

cyclic GMP content of the artery in a $Ca^{++}$-dependent manner without affecting the cyclic AMP content or the effect of $PGE_1$ on it. It is striking that the effect of $O_2$ on cyclic GMP is not prevented by inhibitors of oxidative phosphorylation. $O_2$ was required for demonstration of the $Ca^{++}$-dependent accumulation of cyclic GMP in response to bradykinin, histamine and ionophore. In contrast, serotonin, and also methylene blue and ascorbate like those of serotonin were inhibited by $Ca^{++}$ as well as by $O_2$. These studies have shown that there exist in the umbilical artery, and presumably in other tissues also, at least two separate systems for control of cyclic GMP synthesis that are influenced differently by $Ca^{++}$- and $O_2$-linked processes. Elucidation of the mechanisms through which neurohumoral agents and $O_2$ modify cyclic GMP synthesis and influence the contractility of arterial smooth muscle should aid in understanding the physiological control of perfusion in localized vascular beds and the pathogenesis of certain circulatory disorders.

In studies with human leukocytes we found last year that serotonin, melatonin and related derivatives of tryptamine caused accumulation of cyclic GMP and the effect of these amines was predominantly if not solely on the monocytes. While searching for a functional correlate we have now found in collaboration with the Laboratory of Clinical Investigation, NIAID, that serotonin enhances the responsiveness of these cells to a chemotactic stimulus. Ascorbic acid and carbamylcholine also stimulate monocyte chemotaxis and we have shown that they too increase the cyclic GMP content of human monocytes. All of our findings are consistent with a role for cyclic GMP in modulation of chemotaxis and/or cell movement.

In monocytes (as in the umbilical artery) the effects of serotonin and ascorbic acid on cyclic GMP were unimpaired by the absence of exogenous $Ca^{++}$. The ionophore A23187 which causes $Ca^{++}$-dependent accumulation of cyclic GMP in the artery (and other tissues) did not increase cyclic GMP but caused significant rises in cyclic AMP in monocytes and polymorphonuclear leukocytes. The effect of the ionophore was not dependent upon the presence of $Ca^{++}$ or $Mg^{++}$ in the incubation medium. We are at present evaluating the relationship of the changes in cyclic AMP content to effects of the ionophore on chemotaxis. In any case, it appears that this drug can influence cyclic nucleotide metabolism through more than one mechanism and not all of its effects are necessarily attributable to alterations in $Ca^{++}$ movement.

Cyclic nucleotides have now been implicated in several aspects of leukocyte function relative to inflammatory and immunological responses. In collaboration with the Laboratory of Clinical Investigation, NIAID, we have studied the effects of human transfer factor on cyclic GMP and cyclic AMP in human leukocytes. These preparations transfer cell-mediated immune responsiveness and apparently have both antigen-specific and antigen-independent effects. When mononuclear cells were incubated with dialyzable transfer factor from human mononuclear cells, their cyclic GMP content was rapidly and dramatically increased with no significant change in cyclic AMP. Studies with purified populations of leukocytes established that the response occurred chiefly if not solely in monocytes and neutrophil cyclic GMP was unaffected. The transfer factor preparations contained serotonin

and ascorbic acid in concentrations previously shown to raise cyclic GMP in monocytes. Four fractions separated from dialyzable transfer factor preparations by gel chromatography caused elevation of cyclic GMP in monocytes. The first two contained ascorbate. The others, one of which was the fraction that contained the transfer factor activity, contained no ascorbate or serotonin. Thus it appears that preparations of human transfer factor contain, in addition to ascorbic acid and serotonin, another substance or substances capable of causing accumulation of cyclic GMP in human monocytes. Any or all of these may contribute to their clinical effects perhaps by amplifying subthreshold cellular inflammatory or immune responses.

We have now amassed a number of clues to the ways in which cyclic GMP metabolism may be modulated in intact cells and the types of cellular processes in which it may play a regulatory role. In the next phase of investigation, attention will be focussed on the enzymes that synthesize and degrade cyclic GMP, particularly the guanylate cyclases.

3. Regulation of Hormone-Sensitive Lipase Activity in Fat Cells.

This enzyme which catalyzes the rate-limiting step in triglyceride breakdown continues to resist all attempts in our laboratory and elsewhere at extensive purification. By taking advantage of its substrate affinity we have obtained 20 to 25 fold purification at an early stage but the instability of the enzyme following this procedure has limited its usefulness. In most preparations the lipase tends to be associated with lipids and other proteins in large aggregates. We found last year that by using gel chromatography in the presence of 1 M NaCl the lipase from rat fat could be obtained in a form with an apparent molecular weight of <100,000. In this state it is relatively stable. When subjected to further fractionation, however, by a variety of means, large losses of activity have invariably resulted. Considering that the lipase from adipose tissue of another species might be more amenable to study we carried out exploratory experiments with fat from other rodents and from the chicken. None of the sources tested, however, appeared to offer any advantages over the rat tissue.

As we reported a few years ago, the hormone-sensitive lipase from the rat is inactivated by incubation with ATP, $Mg^{++}$ and ascorbic acid. The requirements for $Mg^{++}$ and ascorbic acid are highly specific but we have recently found that the nucleotide requirement is relatively nonspecific. Thus ADP, GTP, GDP, CTP or CDP can replace ATP and CTP is effective at lower concentrations than are any of the other nucleotides. The related nucleoside monophosphates and cyclic monophosphates are inactive. Until more purified preparations of the lipase are available the significance of the apparent preference for CTP in this reaction remains unclear.

4. Release of Histamine from Mast Cells.

The release of histamine and other vasoactive compounds from mast cells probably plays a role in the pathogenesis of many allergic and inflammatory processes. Dextran causes release of histamine in a genetically determined reaction that in many ways resembles anaphylactic release. It

was used in our earlier work which established that cell desensitization limits the duration of histamine release and is therefore a major determinant of the magnitude of release. Studies now completed confirmed the preliminary findings reported last year that the rate of cell desensitization is also a critical determinant of the amount of histamine that is released as a result of an antigen-antibody reaction.

The systemic reaction of rats to the administration of dextran has been described as resembling anaphylaxis and termed "anaphylactoid". Extensive studies begun last year have, however, failed to demonstrate either precipitating antibodies to dextran or antibodies of the IgE type in the serum of dextran-reactive rats. We have concluded, therefore, that the dextran reaction is due to the existence of natural dextran receptors on mast cells and not to the presence of cytotropic antibodies. A careful comparison of the anaphylactoid reaction to dextran and the reaction to antigen in rats immunized with ovalbumin yielded findings consonant with this view. It seems clear that the anaphylactoid reaction to dextran is not identical with anaphylaxis and its mechanism remains to be elucidated.

Another project related to histamine metabolism was carried out this year in collaboration with members of the Pulmonary Branch, NHLI, who had found that administration of aspirin or sodium salicylate to animals of several species alters the metabolism of histamine such that the formation of 5'-phosphoribosylimidazoleacetate, normally an excretory product, is largely prevented. In an attempt to determine the mechanism of this effect of salicylates, the enzyme responsible for the conversion of imidazoleacetate to its phosphoribosyl derivative was prepared from rat liver. The purified imidazoleacetate phosphoribosyl transferase was inhibited by those salicylate derivatives that are active in vivo in concentrations that would be achieved in tissues. Other anti-inflammatory agents that do not alter the excretion of phosphoribosylimidazoleacetate were not inhibitory.

5. Regulation of Lipid Synthesis in Mammalian Cells.

Work on the hormonal control of lipid metabolism has been largely suspended during the past year while efforts were concentrated on studies of the nature of the metabolic defect in Type II hyperlipoproteinemia as outlined below. In addition, we have investigated the effects of cholesterol feeding on hepatic sterol synthesis in the rat and have found that although it undergoes a rapid decline when cholesterol is added to the diet (as is well known) the depression is not sustained, i.e., with prolonged cholesterol intake the rate of synthesis rises again. This intriguing observation when explained could shed some light on the effects of dietary manipulation on cholesterol metabolism in man.

We found several years ago that cholesterol synthesis in normal human fibroblasts was low when whole serum was present in the medium and increased over a period of hours when the serum was removed or was replaced with lipid-free serum. The rate-limiting step in cholesterol synthesis in many tissues is catalyzed by hydroxymethylglutaryl coenzyme A (HMGCoA) reductase and it was shown that the activity of this enzyme in the normal fibroblasts was

altered in parallel with cholesterol synthesis. Last year we reported that cultured fibroblasts from three patients' homozygous for Type II hyperlipoproteinemia (familial hypercholesterolemia) exhibited an impairment in this negative feedback regulation of HMGCoA reductase activity in response to serum lipoproteins. Cells from one more patient have since been studied. In all of these cell lines (obtained from the Molecular Disease Branch, NHLI) HMGCoA reductase activity was depressed somewhat by whole serum albeit much less than was observed with normal fibroblasts.

Goldstein and Brown, however, had reported a complete lack of effect of serum on HMGCoA reductase activity in fibroblasts from several other patients with familial hypercholesterolemia and it was unclear whether the differences between their findings and ours were due to differences in experimental conditions or were an indication of heterogeneity in the population phenotypically designated as Type II hyperlipoproteinemia. We therefore obtained fibroblasts from one of the patients originally studied by Goldstein and Brown and from another apparently similar patient. The changes in HMGCoA reductase activity in response to serum in those cells and in the cells that we had originally studied were compared. It was found that the defect in feedback regulation in the cells from the NIH patients, although very real, is much less severe than that in the other two cell lines. Thus, it appears that the syndrome clinically described as Type II hyperlipoproteinemia is not the result of a single genetic abnormality. A similar conclusion was reached by Goldstein and coworkers who have recently studied fibroblasts from three patients that behave very much like those from the NIH patients. They have related the metabolic abnormalities in different Type II fibroblasts to defects in the cell surface receptors for low density lipoproteins. In this regard, we have found that the rate of uptake of radio-labeled triglycerides from low density and very low density lipoproteins is only about 10% of normal with cells from the Type II patient that are designated "receptor negative" by Goldstein et al. and about 50% of normal with the apparently less severely defective cells from the NIH patients. These studies which are still in the initial stages are part of a continuing investigation of lipid transport and synthesis in human fibroblasts with the goal of defining in detail the nature of the abnormalities in hypercholesterolemia and other disorders of lipid metabolism.

Project No. Z01 HL 00601-06 LCM
1. Laboratory of Cellular Metabolism
3. Bethesda, Md.

PHS - NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Regulation of Lipid Synthesis in Mammalian Cells

Previous Serial No.: NHLI-29

Principal Investigator: Joel Avigan, Ph.D.

Other Investigator: Marta E. Schreiner, B.S.

Cooperating Units: Molecular Disease Branch, NHLI

Project Description:

The objective of this project was to study hormonal and feedback control of sterol and fatty acid synthesis in cells grown in culture and in tissues of laboratory animals. Cultures of human fibroblasts that were originally obtained from investigators in the Molecular Disease Branch of NHLI as well as other cell lines received from the NIGMS Genetic Mutant Cell Repository were grown in monolayers to confluency and then incubated in minimal essential medium with additions as indicated; e.g., hormones, serum, or fractions thereof. Fatty acid and sterol synthesis were determined by incubating cell cultures with radioactive acetate. The activity of hydroxymethylglutaryl coenzyme A (HMGCoA) reductase was assayed in cell homogenates incubated with HMGCoA followed by isolation of the radioactive mevalonic acid by a method that was previously modified in this laboratory (NHLI-29, 1974). The uptake of triglycerides by fibroblasts from serum lipoproteins was measured following incubation of the cells with medium containing lipoproteins labeled in vitro with radioactive tripalmitin. Rat hepatic microsomal HMGCoA reductase was determined in preparations obtained from animals maintained for 0-3 weeks on diets containing cholesterol and sacrificed at the time of peak activity of the enzyme in the diurnal cycle.

Effects of glucocorticoids. In our previously reported studies (NHLI-276, 1973) it was observed that dexamethasone stimulates fatty acid and nonsaponifiable lipid synthesis in several diploid cell lines and that no such effect occurred in some permanent lines. It was subsequently shown that sterol synthesis in an organized tissue (rabbit aortic media and intima) was also stimulated by 50% following incubation for 2 days in vitro with dexamethasone, 0.1 or 1 μM. Four compounds with glucocorticoid activity in vivo (dexamethasone, hydrocortisone, fluocinolone acetonide and prednisolone) were studied with respect to their effects on lipid synthesis in human skin fibroblasts. At concentrations of 0.1 or 1 μM

all stimulated this process but only prednisolone was active at 0.01 μM. Regulation of sterol synthesis is believed to occur mostly through changes in the activity of HMGCoA reductase - a key enzyme on the biosynthetic pathway. Dexamethasone, 0.1 or 1 μM, did not consistently affect reductase activity in human fibroblasts but 100 μM increased it greatly. On the other hand, in a transformed cell line (L-cells) the HMGCoA reductase was not induced by dexamethasone even at 100 μM, which was consistent with the previously observed lack of stimulation of lipid synthesis in these cells.

Feedback control of cholesterol synthesis in human fibroblasts derived from Type II hyperlipemic homozygous and from normal donors. The previous study was extended to a cell line described by Goldstein and Brown (Nat. Acad. Sci. USA 70, 2809, 1973) as feedback receptor negative. Following incubations under standard conditions in the presence and in the absence of low density serum lipoproteins, we confirmed that in this cell line there is a total absence of effect of LDL on HMGCoA reductase activity. On the other hand, in cells from the four type II homozygous patients studied at the NIH, LDL decreased HMGCoA reductase activity although to a lesser degree than in control cells. The basis for the difference in responsiveness of these cell lines is not clear at the present time, but the abnormal condition may be polygenic and caused by more than one genetic mutation.

Triglyceride uptake by normal and type II homozygous fibroblasts. It was shown that the type II cells take up labeled triglycerides complexed with low density or very low density serum lipoproteins at a slower rate than do normal cells. Further studies concerning the deficiencies in lipid transport are now in progress.

The effect of feeding cholesterol on rat hepatic HMGCoA reductase. It has been repeatedly reported in literature that feeding cholesterol to rats causes a rapid decline in the level of hepatic cholesterol synthesis and of HMGCoA reductase activity. There was a valid interest in exploring the effect of sustained dietary intake of cholesterol on the activity of the enzyme as compared with that of a short-term feeding. It was shown that contrary to expectations the activity of hepatic microsomal HMGCoA reductase was higher following a 2-week period of feeding a diet containing 5% cholesterol than after a period of 3 days. The physiological significance of the rebound in enzyme activity on extended cholesterol feeding is presently unknown.

It is proposed to study further the nature of the abnormality in lipoprotein transport and in the regulatory system affecting cholesterol synthesis in fibroblasts grown from patients with type II hyperlipoproteinemia and also to investigate the transport and metabolism of triglycerides in cells derived from individuals with other types of hyperlipidemia.

Publications:

Avigan, J., Bhathena, S. J., and Schreiner, M. E.: Control of sterol synthesis and of hydroxymethylglutaryl CoA reductase in skin fibroblasts grown from patients with homozygous type II hyperlipoproteinemia. J. Lipid Res. 16: 151-154, 1975.

Keywords:

Cholesterol synthesis - HMGCoA reductase - tissue culture - human skin fibroblasts - glucocorticoids - type II hyperlipoproteinemia - triglyceride uptake - feedback control - arterial tissue - hepatic microsomes.

Project No. Z01 HL 00602-05 LCM
1. Laboratory of Cellular Metabolism
3. Bethesda, Md.

PHS - NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Histamine Release from Mast Cells; Immediate Hypersensitivity

Previous Serial No.: NHLI-30

Principal Investigator: James H. Baxter, M.D.

Other Investigator: Ronald Adamik, B.S.

Project Description:

Objective: to define the mechanisms that control release of mediators from mast cells, and that regulate other cellular functions in the immediate hypersensitivity reaction. Rat peritoneal mast cells are used for the in vitro studies. Prior to harvesting the cells, the donor rats may be immunized with an antigen (egg albumin) and pertussis vaccine in order to sensitize the cells with cytotropic antibody. The release of histamine (and other mediators) by the antigen, and by dextran and other releasing agents is then studied under various conditions. Studies are also made on the systemic reactions of rats to antigen and to dextran.

Role of cell desensitization in controlling histamine release by antigen. Continuing the studies on mast cell desensitization by dextran described last year, histamine release from immunized rat mast cells by antigen was studied in terms of rate of release and duration of release, which together determine the total amount released. With the use of various antigen concentrations and incubation temperatures, release always stopped at about the same time that the cells became desensitized to the antigen. It appeared, therefore, that the rate of desensitization (under the influence of environmental factors) determined the duration of release, and thereby was an important determinant of the total amount of histamine released.

Calcium and phosphatidyl serine (PS) effects in mast cell histamine release by dextran. Histamine release from rat mast cells by dextran (together with 7 µg/ml PS) required greater than 0.1 mM $Ca^{++}$, and maximal release (at pH 7) about 1 mM $Ca^{++}$. Cell desensitization by dextran likewise required $Ca^{++}$. Spontaneous leakage of histamine from the cells was decreased by $Ca^{++}$. Cells suspended for 15 min in $Ca^{++}$-free medium had to be preincubated with $Ca^{++}$ for 10 min (at 25°) before their responsiveness to dextran was maximally restored; responsiveness never returned

to the original level. Histamine release could be stopped short of completion by adding EDTA or glucose, or by diluting the cells (and dextran). $Na^{+}$ and $K^{+}$ were not required for histamine release, and ouabain was without effect on the reaction. $Sr^{++}$, $Ba^{++}$ and $Mg^{++}$ were ineffective in replacing $Ca^{++}$, but did decrease histamine leakage. The studies described above were made with PS in the medium. In the presence of $Ca^{++}$, PS greatly enhanced release by dextran, by increasing the rate of release without affecting the rate of cell desensitization. Some evidence of an interaction between $Ca^{++}$ and PS (in their effects on histamine release) was demonstrated, in that the two agents were strongly synergistic, and when PS was present a greater concentration of $Ca^{++}$ was required for maximal release by dextran.

Anaphylactoid reaction in rat: non-antibody dependence and non-identity with anaphylaxis. Systemic reactions of some nonimmunized animals to injections of certain substances which do not harm most animals are well known. These reactions are often species (or strain) specific; they resemble anaphylaxis, and have been suspected of being due to antibodies. We have investigated the basis of one such reaction, the "anaphylactoid" reaction of the rat to dextran. Not only did we fail to demonstrate precipitating antibodies against dextran in the serum of dextran-reactive (Sprague-Dawley) rats, but also the serum of such rats failed to prepare the skin of non-dextran-reactive (Wistar/Furth) rats for passive cutaneous anaphylaxis or their peritoneal mast cells for histamine release by dextran. The negative results with dextran were obtained in parallel with positive control results with egg albumin after use of serum from rats that had been immunized with egg albumin and pertussis vaccine. Therefore, we conclude that the dextran reaction is a result of natural dextran receptors on the mast cells, and not of the presence of cytotropic antibodies.

A comparison of the anaphylactoid reaction to dextran and the reaction to antigen (egg albumin) in Sprague-Dawley rats that had been immunized with the antigen and pertussis vaccine, indicated that the two reactions were different, and therefore may involve different mechanisms. When compared at approximately equal levels of mortality, the dextran reaction was characterized by severe acral edema, whereas the reaction to antigen exhibited intestinal edema and more severe respiratory disturbances.

We plan to study: (1) release of serotonin and heparin, as well as histamine, from mast cells; (2) interaction of lipids and other substances with mast cell membranes; (3) basis of differences in the rat reactions to dextran and antigen.

Significance to heart and lung research: Histamine and other physiologically active substances which are released from mast cells produce important effects on the small blood vessels and on bronchial smooth muscle.

Publications:

Baxter, J. H. and Adamik, R.: Control of histamine release: effects of various conditions on rate of release and rate of cell desensitization. J. Immunol. 114: 1034-1041, 1975.

Keywords:

Mast cells, histamine release, cell desensitization, dextran, antigen, cytotropic antibody, calcium, phosphatidyl serine, anaphylaxis, anaphylactoid reaction, passive cutaneous anaphylaxis.

Project No. Z01 HL 00603-01 LCM
1. Laboratory of Cellular Metabolism
3. Bethesda, Md.

PHS - NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Regulation of Rat Liver Phosphodiesterases

Previous Serial No.: None

Principal Investigators: Joel Moss, M.D., Ph.D.
Martha Vaughan, M.D.

Other Investigators: Vincent C. Manganiello, M.D., Ph.D.
Sally Stanley, B.S.

Project Description:

Objectives: To define the structural and kinetic characteristics of phosphodiesterases responsible for cyclic nucleotide hydrolysis in rat liver.

Methods: Phosphodiesterase activity is measured by methods previously developed in this laboratory. The enzyme will be purified by affinity chromatography.

Major Findings: A previously identified guanosine cyclic 3',5'-monophosphate-stimulated adenosine cyclic 3',5'-monophosphate phosphodiesterase was partially purified from the 100,000 g supernatant fraction of rat liver. The kinetics of cyclic AMP hydrolysis were consistent with those of an allosteric enzyme displaying positive cooperativity between catalytic sites. In the presence of μmolar cyclic GMP, hydrolysis of μmolar cyclic AMP was stimulated 10-fold. The marked sigmoidicity of the curve relating rate of cyclic AMP hydrolysis to cyclic AMP concentration was not evident when cyclic GMP was present. In addition to cyclic GMP, cyclic IMP and cyclic XMP stimulated cyclic AMP hydrolysis. The $K_a$'s for cyclic IMP and cyclic XMP were approximately one and three orders of magnitude higher than that for cyclic GMP.

The purified phosphodiesterase also catalyzed the hydrolysis of cyclic GMP, the $K_m$ being approximately half that noted for cyclic AMP. Cyclic IMP, cyclic AMP and cyclic XMP accelerated the hydrolysis of cyclic GMP when the latter was present at nmolar concentrations. Cyclic IMP proved to be the most potent effector ($K_a$=1-2 μmolar) followed by cyclic AMP ($K_a$=2-4 μmolar) and cyclic XMP ($K_a$=400 μmolar). Hydrolysis of nmolar cyclic IMP was stimulated by cyclic GMP, cyclic AMP and cyclic XMP in order of increasing $K_a$. The kinetic data suggest that

this phosphodiesterase favors cyclic GMP both as a substrate and an effector. The physiological significance of this is unclear.

Significance to Heart and Lung Research: Control of cyclic GMP and cyclic AMP hydrolysis is important in the action of hormones on the cardiovascular system and in the contraction of smooth muscle in the lung.

Proposed Course: The phosphodiesterase will be further purified and its kinetic and structural characteristics studied.

Publications: None

Keywords:

Phosphodiesterase, cyclic nucleotides, adenosine 3',5'-monophosphate, guanosine 3',5'-monophosphate.

Project No. Z01 HL 00604-01 LCM
1. Laboratory of Cellular Metabolism
3. Bethesda, Md.

PHS - NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Inhibition of Histamine Metabolism by Salicylates

Previous Serial No.: None

Principal Investigators: Joel Moss, M.D., Ph.D.
Maria C. de Mello, Ph.D.
Martha Vaughan, M.D.
Michael A. Beaven, Ph.D.

Cooperating Units: Pulmonary Branch, NHLI
Dr. de Mello is supported by the Brazilian National Research Council

Project Description:

Objectives: To define the mechanism for the in vivo inhibition by salicylates of the formation of the histamine metabolite, 5'-phosphoribosylimidazoleacetate.

Methods: Imidazoleacetate phosphoribosyl transferase was isolated by standard chromatographic and ultracentrifugal procedures. Product identification was based on methods developed by Beaven, et al. (1).

Major Findings: Beaven and coworkers (1) previously demonstrated that the administration of sodium salicylate or aspirin alters the metabolism of histamine in several animal species. The formation of the histamine metabolite, 5'-phosphoribosylimidazoleacetate, was inhibited by these salicylates, but was not affected by other anti-inflammatory agents. In an attempt to define the mechanism of this salicylate effect, the enzyme responsible for the conversion of imidazoleacetate to its phosphoribosyl derivative was purified from rat liver by ultracentrifugation and DEAE-cellulose chromatography. The purified transferase preparation was inhibited by those salicylate derivates active in vivo at concentrations that would be achieved in tissues.

Significance to Heart and Lung Research: Histamine exerts a profound effect on the smooth muscle of the lung. Inhibition of one of the pathways for histamine degradation by salicylates may thus be relevant in the pathogenesis of certain disease states.

Proposed Course: The effects of salicylate metabolites and anti-inflammatory agents on the transferase reaction will be evaluated.

Publications:

(1) Beaven, M.A., Horakova, Z., and Keiser, H.: Inhibition by aspirin of ribose conjugation in the metabolism of histamine. European J. Pharm. 29: 138-146, 1974.

Keywords:

Salicylates, aspirin, anti-inflammatory agents, 5'-phosphoribosylimidazoleacetate, imidazoleacetate phosphoribosyl transferase.

Project No. Z01 HL 00605-02 LCM
1. Laboratory of Cellular Metabolism
3. Bethesda, Md.

PHS - NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Cyclic Nucleotide Metabolism in Human Umbilical Artery

Previous Serial No.: NHLI-36

Principal Investigators: Ronald Clyman, M. D.
Vincent Manganiello, M.D., Ph.D.
Martha Vaughan, M. D.

Other Investigator: Adam Blacksin

Project Description:

Objectives: To determine the effects of divalent cations and oxygen on cyclic nucleotide metabolism in the human umbilical artery.

Methods: Umbilical cords from term (gestational) pregnancies were obtained within 30 min of delivery. Umbilical artery segments were prepared as described by Clyman et al. (1). Variations in incubation medium $Ca^{++}$-content and $O_2$-content were made as described previously. cGMP and cAMP were measured as described in Ref. (2).

Major Findings: We have previously demonstrated that in term gestational human umbilical artery segments incubated in room air at 37°C, histamine, acetylcholine, bradykinin, $K^+$ and serotonin (agonists that cause contraction) cause accumulation of cGMP without altering the content of cAMP; prostaglandin $E_1$ ($PGE_1$), which relaxes the artery, caused cAMP accumulation without affecting the cGMP content.

Calcium ($Ca^{++}$) appears to be important in the regulation of cyclic nucleotide content in several tissues. In the umbilical artery the control of cAMP content by $PGE_1$ was independent of $Ca^{++}$. After incubation in $Ca^{++}$-free medium, the cGMP content of the artery segments was decreased by 50% and was unaffected by histamine, acetylcholine, bradykinin and $K^+$. Readdition of $Ca^{++}$ (2.7 mM) or $Sr^{++}$ (3.6 mM) to the medium partially restored the basal cGMP content and the agonist effects on the cGMP content. However, $Sr^{++}$ was not as effective as $Ca^{++}$ in this regard. Ionophores A23187 and X537A (agents that facilitate $Ca^{++}$ movement through membranes) mimicked the effects of these $Ca^{++}$-dependent agonists on cGMP content. Incubation with the phosphodiesterase inhibitor 3-isobutyl-1-methyl xanthine (0.1 mM) increased both the basal content of cGMP and the histamine

induced accumulation 3-fold. This effect was dependent on the presence of $Ca^{++}$ also. Accumulation of cGMP induced by serotonin, on the other hand, was not diminished in $Ca^{++}$-depleted arteries and, in fact, seemed to be inhibited by 2.7 mM $Ca^{++}$. Thus agonists controlling cGMP accumulation appear to act through two different mechanisms: one $Ca^{++}$-dependent, the other $Ca^{++}$-inhibited.

$O_2$ acts in at least two separate ways to initiate closure of the umbilical artery at birth. $O_2$, itself, apparently directly induces constriction; it plays further a "permissive" role in the action of other chemical agents that cause contraction. We found that $O_2$ increased the cGMP content of the artery in a $Ca^{++}$-dependent manner without affecting cAMP content. Inhibitors of oxidative phosphorylation (oligomycin and 2,4-dinitrophenol) did not inhibit this effect of $O_2$. $O_2$ was required for demonstration of the $Ca^{++}$-dependent accumulation of cGMP in response to bradykinin, histamine, and ionophore A23187. The effect of isobutyl methyl xanthine on basal content and on the bradykinin-induced accumulation was also dependent on the presence of $O_2$. Methylene blue and sodium ascorbate caused cGMP accumulation in $O_2$-deprived arteries. Their effects were not diminished in $Ca^{++}$-depleted arteries and, in fact, seemed to be inhibited when 2.7 mM $Ca^{++}$ was present in the medium. The effects of these agents and of serotonin on cGMP, which were inhibited by $Ca^{++}$, were also inhibited by $O_2$. These non-$Ca^{++}$-, non-$O_2$-dependent agonists (methylene blue, ascorbate, and serotonin) did not, however, permit demonstration of the effects of the $Ca^{++}$- and $O_2$-dependent agonists on $O_2$-deprived arteries. It appears that there are in the umbilical artery at least two separate mechanisms for control of cGMP synthesis that are influenced differently by $Ca^{++}$- and $O_2$-linked processes.

Significance to Heart and Lung Research: Elucidation of the mechanisms by which neurohumoral agents and $O_2$ influence the contractility of arterial smooth muscle should aid in understanding the physiological control of perfusion in localized vascular beds and the pathogenesis of certain circulatory disorders.

Proposed Course: Studies with the human umbilical artery will be terminated. The control of cyclic GMP metabolism and particularly of guanylate cyclase activity will be further investigated using cultured arterial smooth muscle cells.

Publications:

(1) Clyman, R.I., Sandler, J.A., Manganiello, V.C., and Vaughan, M. Guanosine 3',5'-monophosphate and adenosire 3',5'-monophosphate content of human umbilical artery. J. Clin. Invest. 55: 120-125, 1975.

(2) Clyman, R.I., Blacksin, A.S., Sandler, J.A., Manganiello, V.C., and Vaughan, M. Role of $Ca^{++}$ in the regulation of cyclic nucleotide content in human umbilical artery. J. Biol. Chem. 1975, in press.

Keywords:

Umbilical artery, calcium, cyclic nucleotides, oxygen, ascorbate, methylene blue, serotonin, bradykinin, histamine.

Project No. Z01 HL 00606-04 LCM
1. Laboratory of Cellular Metabolism
3. Bethesda, Md.

PHS - NIH
Individual Project Report
July 1, 1974 through June 30, 1975

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| Project Title: | Cyclic Nucleotide Metabolism in Cultured Cells |
| Previous Serial No.: | NHLI-31 and 32 |
| Principal Investigators: | Vincent C. Manganiello, M.D., Ph.D.<br>Joel Moss, M.D., Ph.D.<br>Martha Vaughan, M.D. |
| Other Investigators: | Betty Hom, B.S.<br>Sally Stanley, B.S. |
| Cooperating Units: | P. Fishman, Developmental and Metabolic Neurology Branch, NINDS |

Project Description:

Objectives: To study control of cAMP and cGMP metabolism in cultured cells. In the past year human fibroblasts have been used to investigate the mechanism of action of choleragen or adenylate cyclase and the part played by gangliosides in the cell surface receptor for choleragen.

Methods: Measurement of cAMP by method of Gilman (Proc. Nat. Acad. Sci. 67: 305, 1970); adenylate cyclase by the method described by us (manuscripts in preparation); phosphodiesterase by our published methods (Proc. Nat. Acad. Sci. 69: 269, 1972; 70: 3830, 1973).

Major Findings:

Effects of choleragen on adenylate cyclase activity. Within 30 min after addition of choleragen cAMP content of cultured human fibroblasts was increased. Coincident with the increase in cAMP content was an apparent alteration in response to isoproterenol and $PGE_1$. At all concentrations of isoproterenol, the increment in cAMP produced during a 10 min incubation with isoproterenol was enhanced in the toxin-treated cells. In the presence of maximally effective concentrations of $PGE_1$, the increment in cAMP produced by $PGE_1$ was either similar in both control cells or toxin-treated cells or actually lower in the toxin-treated fibroblasts. At lower concentrations of $PGE_1$, accumulation of cAMP was enhanced in the toxin-treated cells.

After incubation with cholera toxin, although basal adenylate cyclase activity of fibroblast homogenates was increased, no enhancement

of the response to isoproterenol or $PGE_1$ was observed. To elucidate mechanism of interaction of cholera toxin with adenylate cyclase, calls were incubated with $I^{125}$ cholera toxin; membrane fractions were prepared and solubilized with the nonionic detergent Lubrol PX. Although both cyclase activity and $I^{125}$ toxin were found to co-chromatograph, such studies did not establish any definite association between toxin and adenylate cyclase.

$GM_1$ ganglioside and the action of choleragen. Human fibroblasts lack capacity to synthesize $GM_1$ ganglioside. Since $GM_1$ ganglioside is thought to serve as the cell surface receptor for cholera toxin, and since the fetal calf serum used in our growth medium contains high concentrations of $GM_1$, we have assumed that fibroblasts incorporate exogenous $GM_1$ into their cell membranes, and this binding accounts for the responsiveness of these cells to cholera toxin. We have now shown that cells grown in chemically defined medium, in the absence of serum, do not respond to cholera toxin. In current studies with replacement of $GM_1$ ganglioside we expect to learn more about the nature of the choleragen receptor and its relation to adenylate cyclase.

Significance to Heart and Lung Research: Studies of the regulation of cAMP and cGMP metabolism in homogeneous populations of cultured cells should aid in understanding the nature of cellular regulatory processes through which hormones, prostoglandins and other humoral agents act on the lung and cardiovascular system.

Proposed Course: The mechanism of action of choleragen will be investigated in human fibroblasts and other types of cells, especially those that can be grown in chemically defined, serum-free medium. We also plan to study the interrelationships between cAMP and cGMP metabolism in cultured cells, especially cells of smooth muscle origin.

Publications:

Vaughan, M.: Effects of choleragen and fluoride on adenylate cyclase. In Dumont, J.E., Brown, B., and Marshall, N. (Eds.): Regulation of Function and Growth of Eukaryotic Cells by Intracellular Cyclic Nucleotides, New York, Plenum Publ. Corp., 1975, in press.

Keywords:

Tissue culture, cAMP, cGMP, choleragen, $GM_1$ ganglioside, adenylate cyclase, receptor isoproterenol, $PGE_1$, L-2071 cells.

Project No. Z01 HL00607-02 LCM
1. Laboratory of Cellular Metabolism
3. Bethesda, Md.

PHS - NIH
Individual Project Report
July 1, 1974 through June 30, 1975

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| Project Title: | Cyclic Nucleotide Metabolism in Human Leukocytes |
| Previous Serial No.: | NHLI-35 |
| Principal Investigators: | Jeffrey A. Sandler, M. D.<br>Martha Vaughan, M. D. |
| Other Investigators: | Vincent C. Manganiello, M.D., Ph.D.<br>Ronald I. Clyman, M. D. |
| Cooperating Unit: | Dr. John I. Gallin<br>Dr. Charles H. Kirkpatrick<br>Laboratory of Clinical Investigation, NIAID |

Project Description:

Objectives: To assess (1) the relationship between chemotaxis and cyclic nucleotide content in human polymorphonuclear and mononuclear leukocytes, (2) the effects of dialyzable transfer factor on cyclic GMP content of leukocytes, (3) the relationship between cyclic AMP and cyclic GMP content in human monocytes, and (4) the role of divalent cations in cyclic nucleotide generation in leukocytes.

Methods: Cells were separated and incubated and cyclic nucleotides extracted, purified and assayed by standard methods. Chemotaxis was evaluated by a modification of the Boyden chamber procedure.

Major Findings:

1. Chemotaxis and cyclic nucleotides: Serotonin, ascorbic acid and carbamylcholine enhanced the responsiveness of human monocytes to a chemotactic stimulus (endotoxin-treated serum). These agents caused significant accumulation of cyclic GMP in monocytes providing further evidence for a relationship between intracellular cyclic GMP and monocyte movement. PMN leukocyte chemotaxis was also enhanced by these agents although significant increases in cyclic GMP were not demonstrated.

2. Effect of dialyzable transfer factor on cyclic GMP in human monocytes:

Incubation of human leukocytes for 5 min with dialysates of leukocyte lysates that contained transfer factor or with leukocyte dialysates devoid of transfer factor caused a rise in their cGMP content

with little change in cAMP. The accumulation of cGMP occurred predominately if not exclusively in monocytes. Substances that increased monocyte cGMP could be obtained from several cell populations including mononuclear cells from Hypaque-Ficoll gradients, plastic-adherent monocytes, non-adherent lymphocytes and neutrophils, but were not present in dialysates of leukemic lymphocytes from patients with the Sezary syndrome.

Dialysates of lysed mononuclear cells contained serotonin, ascorbate and an unidentified cholinergic activity as well as transfer factor. Passage of these dialysates through a column of Sephadex G-25 yielded four fractions that increased leukocyte cGMP. Two of these fractions contained ascorbate; two other active fractions, including the one that caused conversion of delayed skin tests, did not contain detectable ascorbate or serotonin. When a dialysate of lysed neutrophils which contained no transfer activity was passed over the same column, only the fractions that contained ascorbate caused accumulation of cyclic GMP in mononuclear cells.

These observations are consistent with the possibility that some aspects of transfer factor activity may be effected through cyclic GMP dependent processes.

3. Relationship between cAMP and cGMP in human monocytes: When the cGMP content of monocytes was increased by serotonin or ascorbic acid and the cAMP content was elevated by $PGE_1$, polystyrene beads or the ionophore A23187, there was no change in basal cGMP content but the increment produced by serotonin or ascorbic acid was markedly reduced. Serotonin did not interfere with the effects of $PGE_1$ on cAMP. We have not yet defined the mechanism by which agents that raise cAMP interfere with the accumulation of cGMP in monocytes.

4. Role of $Ca^{++}$ and $Mg^{++}$ in cyclic nucleotide metabolism. The effects of serotonin and ascorbic acid on accumulation of cyclic GMP in human monocytes are apparently independent of extracellular $Ca^{++}$ and $Mg^{++}$. This is in contrast to observations with other tissues in which accumulation of cGMP in response to several agents does not occur in the absence of exogenous $Ca^{++}$.

Ionophore A23187, an agent reported to enhance calcium movement across biologic membranes (and to cause accumulation of cGMP in other tissues) did not increase cGMP but caused a significant accumulation of cAMP in both monocytes and polymorphonuclear leukocytes. This effect of the ionophore did not require the presence of extracellular calcium or magnesium.

Significance to Heart and Lung Research: Phagocytosis is a fundamental cellular function relevant to host defense and the pathogenesis of inflammatory and degenerative processes in all tissues including heart, lung and blood vessels. Information concerning cyclic GMP, the factors that control its metabolism and the role that it plays in cell physiology is at present fragmentary. Available data suggest, however, that it may be of especial importance in the latter tissues.

Proposed Course: The guanylate cyclase and phosphodiesterases of monocytes will be assayed and the effects of cAMP on these enzymes investigated.

Publications:

Sandler, J. A., Clyman, R. I., Manganiello, V. C., and Vaughan, M.: The effect of serotonin (5-hydroxytryptamine) and derivatives on guanosine 3',5'-monophosphate in human monocytes. J. Clin. Invest. 55: 431-435, 1975.

Vaughan, M.: Metabolism of 3',5'-guanosine monophosphate in vascular smooth muscle, leukocytes, and lung. In Dumont, J.E., Brown, B., and Marshall, N. (Eds.): Regulation of Function and Growth of Eukaryotic Cells by Intracellular Cyclic Nucleotides, New York, Plenum Publ. Corp., 1975, in press.

Keywords:

Leukocytes, polymorphonuclear leukocytes, monocytes, cGMP, cAMP, dialyzable transfer factor, calcium ionophore, serotonin, ascorbic acid, chemotoxis.

Project No. Z01 HL 00608-02 LCM
1. Laboratory of Cellular Metabolism
3. Bethesda, Md.

PHS - NIH
Individual Project Report
July 1, 1974 through June 30, 1975

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|---|---|
| Project Title: | Regulation of Cyclic AMP Phosphodiesterase Activity |
| Previous Serial No.: | NHLI-33 |
| Principal Investigators: | C. J. Lovell-Smith, M.D., Ph.D.<br>Martha Vaughan, M. D. |
| Other Investigators: | V. C. Manganiello, M.D., Ph.D.<br>Ferol Lieberman, M.S. |

Project Description:

In order to clarify the complex mechanisms through which cyclic nucleotide phosphodiesterase activity is regulated, we have attempted to: (1) reproduce the in vivo effects of triiodothyronine ($T_3$) on fat cell phosphodiesterase in an in vitro system; (2) solubilize and purify the membrane-bound fat cell phosphodiesterase(s) that is subject to control by insulin, glucocorticoids, cyclic AMP and $T_3$; and (3) use choleragen to modify phosphodiesterase in fat cells. Phosphodiesterase, adenylate cyclase and cyclic AMP were assayed by standard methods.

1. Effects of $T_3$ on fat cells. In preliminary studies last year it appeared that these might be related to a decrease in adenylate cyclase activity but further work has shown that it is probably secondary to the increased activity of a high affinity particulate phosphodiesterase in the fat cells from thyroidectomized rats. Treatment of these animals for 4 days with $T_3$ reversed the changes in phosphodiesterase and restored responsiveness of the fat cells to isoproterenol. It is probably fortuitous that these effects of $T_3$ closely resemble those produced by the addition of $T_3$ to fat cells in vitro. The latter apparently pharmacological effects of $T_3$ are immediate and rapidly reversible and the concentrations required are several orders of magnitude greater than those found in blood.

In order to prove that the results of $T_3$ treatment in vivo are due to a direct effect on fat cells (not secondary to some other hormonal changes resulting from $T_3$ administration) it will be necessary to demonstrate this in vitro. As this presumably physiological effect of $T_3$ is evident only after many hours we carried out studies with fat cells (or fragments of tissue) incubated for one or more days under several different conditions. In all instances, unfortunately, the fat cells failed to retain normal metabolic responsiveness and no effects of $T_3$ were demonstrated.

Currently, we are attempting to study the effect of low concentrations of $T_3$ in cultured cells particularly those that will grow in the absence of serum, or in the presence of serum from hypothyroid animals. As yet, there is no evidence that $T_3$ under these circumstances affects the phosphodiesterases (soluble or particulate) of the lines under study.

2. Solubilization of phosphodiesterase from fat cells. A large number of detergents and other compounds that were tested failed to solubilize the enzyme and/or caused extensive losses of activity. We have recently found that the high affinity particulate phosphodiesterase activity can be solubilized in essentially 100% yield using a combination of BRIJ 30, 0.1%, and 1 M NaCl. It is hoped that the enzyme or enzymes will now be susceptible to purification by standard techniques of ion-exchange, affinity and gel chromatography.

3. Effects of choleragen on fat cells. As first demonstrated in this laboratory several years ago, choleragen increases lipolysis in fat cells after a delay of about one hour. Measurements of fat cell cyclic AMP content in similar experiments revealed that it was significantly elevated only after 4 hr of exposure to choleragen; i.e., well after lipolysis was stimulated. However, by carrying out incubations in the presence of theophylline to inhibit cyclic AMP degradation, effects of choleragen on fat cell cyclic AMP were demonstrable as early as one hour. The conclusion that adenylate cyclase was activated by choleragen within one hour was confirmed by direct assay of the enzyme in particulate fractions and cyclase activity continued to rise during the second and third hours of exposure to choleragen. The particulate phosphodiesterase activity was also increased by choleragen consistent with our earlier observations that when the fat cell cyclic AMP content is elevated (whether by increasing its rate of synthesis or decreasing degradation) phosphodiesterase activity is enhanced.

In relation to the mechanism of action of choleragen it is notable that the magnitude of isoproterenol stimulation is the same with the choleragen-activated adenylate cyclase as it is with the enzyme from control cells.

Significance to biomedical research: It is likely that the phosphodiesterase of many tissues is under regulation by several factors, including hormones. This will probably play an increasingly important role in studies of the cyclic AMP system. The study of thyroid hormones contributes to the relatively little that is known of the mode of action of these hormones.

Proposed course: (a) to define the effect of $T_3$ on phosphodiesterase activity in an *in vitro* system, and (b) to purify and characterise the low $K_m$ particulate phosphodiesterase of rat adipocytes.

Publications:

Vaughan, M.: Regulation of 3',5'-adenosine monophosphate phosphodiesterase activity. In Dumont, J.E., Brown, B., and Marshall, N. (Eds.): Regulation of Function and Growth of Eukaryotic Cells by Intracellular Cyclic Nucleotides, New York, Plenum Publ. Corp., 1975, in press.

Keywords:

Phosphodiesterase, isolated fat cells, triiodothyronine, cyclic AMP, cholera toxin (choleragen), adenylate cyclase, enzyme solubilization.

Project No. Z01 HL 00609-07 LCM
1. Laboratory of Cellular Metabolism
3. Bethesda, Md.

PHS - NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Regulation of Hormone-Sensitive Lipase Activity

Previous Serial No.: NHLI-34

Principal Investigators: Su-Chen Tsai, Ph.D.
Martha Vaughan, M. D.

Other Investigators: None

Project Description:

Objectives: (1) To purify the fat cell hormone-sensitive lipase and the enzymes that regulate its activity. (2) To elucidate the mechanisms of lipase activation and inactivation.

Methods: Assay of lipase activity using $^{3}$H-glyceryl trioleate as substrate, precipitation of the unhydrolyzed substrate with 5% trichloroacetic acid and radioassay of $^{3}$H-glycerol in the supernatant fluid. Fractionation and purification of hormone-sensitive lipase using ammonium sulfate precipitation, gel chromatography and disc gel electrophoresis.

Major Findings:

1. Purification of the lipase from rat adipose tissue. The ammonium sulfate-precipitated lipase was incubated for 5 min at 30° with an emulsion of triolein and mixed phospholipids. After centrifugation about 60% of the lipase activity and 3 to 5% of the protein was associated with the floating lipid layer. When emulsions were made with pure triolein or with phospholipids alone <10% of the lipase was bound to them. The mixed emulsions of phospholipids and triolein similar to that used as a substrate for the enzyme were most efficient for separating the lipase from other proteins in the solution of the ammonium sulfate fraction. Lipase activity could be recovered from the emulsion after removal of lipids with acetone-ether extraction but yields were low. Dissociation of the lipase by incubation of the emulsion in buffer containing 1 M NaCl was more effective but unfortunately the preparations obtained were relatively unstable in subsequent purification steps. It was possible to show that most of the lipase activity in these preparations behaved as a molecule of <100,000 when chromatographed in the presence of 1 M NaCl. We had previously found that in the presence of 1 M NaCl most of the ammonium sulfate-precipitated lipase was dissociated to that size whereas gel chromatography in the absence of NaCl yields lipase activity distributed through a heterogenous family of very large molecules or aggregates.

2. The lipase in adipose tissue from chickens. We considered that the lipase from adipose tissue of species other than the rat might be more amenable to purification and study. There have been a few reports concerning the hormone sensitive lipase in chicken fat and this tissue would offer obvious practical advantages. We found, however, in exploratory experiments that the specific activity of the ammonium sulfate-precipitated lipase from chicken fat was only 20% of that of preparations from rat fat and the chicken enzyme appeared to offer no particular advantages. In particular, inactivation of the chicken lipase with ATP, $Mg^{++}$ and ascorbate was not demonstrable.

3. Cholesteryl esterase activity in lipase preparations. We had found that the ammonium sulfate-precipitated lipase hydrolyzed cholesteryl esters as well as triglycerides. In an attempt to determine whether the same enzyme acted on both substrates activities against triolein and cholesteryl oleate were compared under a variety of conditions known to activate or inactivate the lipase. The effects inhibitors were also compared. These studies were complicated by the fact that the apparent cholesterol esterase activity could be varied widely by very minor changes in the method of substrate preparation or in time elapsed between ints preparation and use. All of the data, however, were consistent with the conclusion that two different enzymes act on the two substrates.

4. Inactivation of the rat lipase. As we reported a few years ago, the hormone-sensitive lipase from the rat is inactivated by incubation with ATP, $Mg^{++}$ and ascorbic acid. The requirements for $Mg^{++}$ and ascorbic acid are highly specific but we have recently found that the nucleotide requirement is relatively nonspecific. Thus ADP, GTP, GDP, CTP or CDP can replace ATP and CTP is effective at lower concentrations than are any of the other nucleotides. The related nucleoside monophosphates and cyclic monophosphates are inactive. Until more purified preparations of the lipase are available the significance of the apparent preference for CTP in this reaction remains unclear.

Significance to Heart and Lung Research: Availability of plasma FFA, an important energy substrate for heart, is regulated via the hormone sensitive lipase activity of adipose tissue. Purification of the lipase and the inactivating enzyme are essential before the mechanisms through which its activity is regulated can be elucidated.

Proposed Course: Purification of the hormone sensitive lipase from adipose tissue will be continued along with studies of the ATP, $Mg^{++}$, ascorbate-dependent inactivation of the enzyme.

Publications: None

Keywords: cholesterol esterase; lipase, hormone-sensitive; lipase inactivation; enzyme regulation.

Annual Report of the
LABORATORY OF CHEMICAL PHARMACOLOGY
National Heart and Lung Institute
July 1, 1974 through June 30, 1975

# DRUG METABOLISM AS A CAUSE OF DRUG TOXICITY

Many organic compounds are transformed in the body to potent alkylating and arylating metabolites that combine covalently with various tissue components, including proteins, lipids and nucleic acids. During the past few years this laboratory has been developing an integrated approach for determining 1) whether these chemically reactive metabolites mediate the different toxicities caused by their parent substances, 2) whether there is a dose threshold for the toxicity and the covalent binding of the chemically reactive metabolites, 3) the mechanisms of formation and inactivation of the reactive metabolites, 4) whether enzymes present in a given tissue account for the formation of the metabolite that becomes covalently bound in that tissue, 5) the mechanisms by which various treatments alter the formation and elimination of the reactive metabolites and thereby change the incidence and severity of the toxicity and 6) the conditions under which extrapolation of data obtained in vitro to living animals will be reasonably valid and when it won't.

To evaluate the approach the laboratory searches for drugs and other foreign compounds that cause tissue lesions and determines whether radiolabeled toxicants are covalently bound to macromolecules in target tissues. It then studies whether the amount of covalently bound metabolite in the target tissue is approximately proportional to the dose of the toxicant and whether various treatments that alter the activity of various enzymes that are known to metabolize foreign compounds cause parallel changes in the amount of covalently bound metabolite in the tissues and in the incidence and severity of the tissue lesion. If the changes in covalent binding and the tissue lesion do not parallel each other, the covalent binding of double-labeled derivatives of the foreign compound and its conjugates is studied to determine whether only a part of the molecule of the toxicant or its conjugate becomes covalently bound. Concurrent studies in vitro aid in elucidating the enzyme that catalyzes the formation of the reactive metabolite, the part of the toxicant's molecule that is activated and whether enzymes in the target tissue can account for the amount of reactive metabolite that becomes covalently bound in the target tissues. Concurrent studies on the pharmacokinetics of the toxicant in vivo and on the distribution of its urinary metabolites aid in elucidating possible mechanisms for dose thresholds for covalent binding and in resolving apparent discrepancies between in vitro and in vivo results. The laboratory is also testing the validity of a simple mathematical model, based on irreversible kinetics, as an aid in resolving possible mechanisms by which treatments may alter covalent binding and toxicity.

With this integrated approach the laboratory has discovered that the liver necrosis caused by halogenated benzenes, acetaminophen and furosemide in animals occursonly after threshold doses are exceeded and that these dose thresholds are due to dose dependent changes in the metabolism of the toxicants. It has also discovered that the incidence of liver damage caused by

other drugs and foreign compounds, including carbon tetrachloride, isoniazid and iproniazid, does not depend on a threshold dose but is approximately proportional to the dose of the toxicant. In addition, the laboratory has discovered why a given treatment may increase the incidence of toxicity caused by a given toxicant at one dose but decreases it at another or increases it in one animal species but decreases it in another. We are thus gaining a better understanding of the mechanisms by which organic compounds cause tissue lesions and of the effects of various treatments and different dosage schedules on drug-induced toxicities.

Isoniazid and related drugs

Programs in which isoniazid was used prophylatically to prevent tuberculosis were stopped because about 1% of the patients manifested a hepatitis-like syndrome which in some cases resulted in death. Last year, we reported evidence that the hepatitis caused by isoniazid occurred predominately in patients that acetylated the drug rapidly and suggested that the hepatitis may be caused by a chemically reactive metabolite formed by the following sequence of events: 1) Isoniazid is first acetylated to form acetylisoniazid which in turn is hydrolyzed by an amidase to isonicotinic acid and acetyl hydrazine. 2) The acetyl hydrazine then is hydroxylated to form a chemically reactive metabolite by a cytochrome P-450 enzyme in liver microsomes. In accord with this view, considerably more isoniazid is excreted as isonicotinic acid in fast acetylators of the drug than in slow acetylators. But there was no difference between fast and slow acetylators in the proportion of the dose of acetylisoniazid excreted into urine as isonicotinic acid. Thus, the increase in acetylisoniazid formation by fast acetylators accounts for the increase in the formation of isonicotinic acid and acetyl hydrazine.

It also seems likely that the liver necrosis observed in patients receiving iproniazid may be caused by a similar sequence of events, that is: 1) iproniazid is hydrolyzed to isonicotinic acid and isopropyl hydrazine and 2) the isopropyl hydrazine in turn is converted to a chemically reactive metabolite by a cytochrome P-450 enzyme in liver endoplasmic reticulum.

The view that the liver damage caused by isoniazid and iproniazid is mediated by acetyl hydrazine and isopropyl hydrazine rather than isonicotinic acid is supported by the following facts: 1) The doses required to produce liver necrosis in rats are much smaller with acetyl hydrazine or isopropyl hydrazine than with acetyliproniazid or iproniazid. 2) The acetyl group of acetylisoniazid is covalently bound in rat liver to a greater extent than is the isonicotinic acid group after administration of doubly labeled acetylisoniazid. 3) Changes in the amount of the acetyl group of acetylisoniazid and acetyl hydrazine and in the amount of the isopropyl group of isopropyl hydrazine that become covalently bound to liver protein *in vivo* parallel changes in the severity of the liver necrosis caused by the pretreatment of animals with phenobarbital or cobaltous chloride.

The identities of the reactive metabolites of acetyl hydrazine and isopropyl hydrazine remain to be determined. *In vitro* experiments have confirmed the view that both acetyl hydrazine and isopropyl hydrazine are converted to their reactive metabolites by a cytochrome P-450 enzyme in liver endoplasmic

reticulum. Experiments with double-labeled acetyl hydrazine have revealed that the acetyl group of the reactive metabolite probably becomes covalently bound intact and that most of it is converted to acetate. Thus, the reactive metabolite is probably an N-hydroxyl derivative of acetyl hydrazine. In vivo the acetate is converted to carbon dioxide. Indeed, changes in the formation of radiolabeled carbon dioxide in vivo after the administration of acetyl-labeled acetylisoniazid or acetyl hydrazine parallel changes in the covalent binding of the acetyl group to liver proteins. The formation of radiolabeled carbon dioxide may thus be used as an indirect measure of the formation of the reactive metabolite of acetyl hydrazine. Experiments with $^{14}$C-1, $^{3}$H-2-isopropyl hydrazine indicate that the isopropyl group of its reactive metabolite is also covalently bound to liver proteins intact and thus the reactive metabolite is probably an N-hydroxyl derivative of isopropyl hydrazine. Some of the reactive metabolite is converted to propane. But studies in vivo have revealed that phenobarbital pretreatment decreases the formation of propane from isopropyl hydrazine even though it increases covalent binding of the reactive metabolite. The relationships between covalent binding of the reactive metabolite and propane formation therefore need to be clarified.

## OTHER STUDIES RELATED TO THE METABOLISM AND TOXICITY OF DRUGS

Chloramphenicol (d-(-)-threo-1-(p-nitrophenyl)-2-(dichloroacetamido)-1,3-propanediol) - Previous studies have shown that chloramphenicol in vivo is covalently bound predominately to proteins in liver, bone marrow and plasma, but the mechanism of activation was unclear. During the past year, chloramphenicol was labeled with $^{3}$H in the 1-position of the propanediol and with $^{14}$C in the dichloroacetyl group. In vitro studies revealed that about 20% more of the $^{3}$H-labeled derivative was covalently bound to liver microsomes than was the $^{14}$C-labeled derivative. By contrast, in vivo studies revealed that 5-10 times more of the $^{14}$C-label was covalently bound to tissue proteins than was the $^{3}$H-label, suggesting that the chloramphenicol was cleaved either before or after it became covalently bound. In either case, most of the covalent binding occurring in vivo does not appear to be mediated by the reduction of the nitro group. In this regard, it may be important that $^{14}$C-dichloroacetic acid is also covalently bound extensively to tissue proteins in vivo.

Nitrobenzene and other nitrobenzenes - Nitrobenzenes are known to cause methemoglobinemia presumably through their reduction to nitroso or hydroxylamine derivatives. Although nitro compounds may be reduced by several different mammalian enzymes including NADPH cytochrome c reductase, cytochrome P-450, xanthine oxidase and aldehyde oxidase, they also may be reduced by intestinal flora. Indeed, the finding that nitrobenzene given either intraperitoneally or orally does not cause methemoglobinemia in germ-free rats or in those treated with antibiotics suggests that the reduction of nitrobenzene in vivo is predominately by intestinal bacteria. Surprisingly, in rats kept under ordinary laboratory conditions, the methemoglobinemia caused by nitrobenzene is greater when it is administered intraperitoneally than when it is given orally. Thus, intraperitoneal administration of drugs does not preclude the possibility that they might be metabolized by intestinal bacteria even when

the drugs are not excreted in bile.

Role of cytochrome $b_5$ in the formation of superoxide by cytochrome P-450 systems - Studies on the relative rates of oxidation of NADH and NADPH have revealed that at least 2/3 of the electrons required for the reduction of oxygenated-cytochrome P-450-substrate complexes in liver microsomes are mediated by cytochrome $b_5$. The finding that an anti-cytochrome $b_5$ antibody preparation does not inhibit NADPH oxidation as much as it inhibits drug metabolism, however, suggests that the oxygenated cytochrome P-450-substrate complexes may decompose when the rate of reduction of the complexes to "active oxygen" cytochrome P-450-substrate complexes is the rate-controlling step in drug metabolism. In accord with this view, superoxide is formed by the cytochrome P-450 system in liver microsomes and its rate of formation is increased by the anti-cytochrome $b_5$ antibody.

Pretreatment of male rats with either spironolactone or pregnenolone-16α-carbonitrile (PCN) also leads to the formation of cytochrome P-450 systems in which the rate-limiting step is apparently the reduction of the oxygenated-cytochrome P-450 complex. After the addition of substrates to liver microsomes from rats pretreated with these substances, the rate of substrate-dependent NADPH oxidation is greater than the rate of drug metabolism. This extra NADPH oxidation is apparently due to the formation of superoxide, because more superoxide is formed by these liver microsomes than is formed by microsomes from phenobarbital pretreated rats. Moreover, studies on the rate of reduction of cytochrome $b_5$ by NADH or NADPH indicate that these reactions are slower in liver microsomes from PCN treated rats than in those from phenobarbital pretreated rats.

α-Methyl dopa - Large doses of α-methyl dopa (> 250 mg/kg) in rats cause a mild hepatic injury characterized by diffuse acidophilic bodies, without depletion of glutathione or increases in diene conjugation of phospholipids. *In vitro* studies have shown that α-methyl dopa is converted to a chemically reactive metabolite by enzyme systems, such as cytochrome P-450 in liver microsomes and xanthine oxidase. Since superoxide dismutase and various catechols inhibit the covalent binding of radiolabeled α-methyl dopa, in the presence of either enzyme, it seems probable that the reactive metabolite is formed indirectly by the superoxide produced by these enzymes rather than by a direct action of these enzymes on α-methyl dopa. Whether the formation of chemically reactive metabolites of α-methyl dopa *in vivo* are mediated by superoxide, however, remains to be determined.

Paraquat - This herbicide causes edema and necrosis in pulmonary alveoli followed by interstitial fibrosis and death. Although it is commonly believed that the toxicity is mediated by superoxide formed during the autoxidation of the reduced form of the herbicide, we have not been able to demonstrate that lipid peroxidation, which is caused by superoxide, occurs either in living animals or in lung slices. Last year, we reported that the toxicity might be affected by altering β-adrenergic responses in lung because the lethal effects of the herbicide are potentiated by isoproterenol and are decreased by propranolol. However, the relationships between the β-adrenegric system in lung and paraquat toxicity are obscure because paraquat causes a decrease rather than an increase in cyclic AMP. Moreover, the pro-

tective effects of propranolol and similar β-adrenergic blocking agents may be due largely to the inhibition of the paraquat active transport system in lung slices discovered by Rose et al. (Nature 252:314, 1974) even though propranolol did not appear to decrease appreciably the uptake of paraquat into rat lung.

## PHYSIOLOGICAL CONTROL MECHANISMS

Cyclic nucleotide formation - Last year, we reported that the guanylate cyclase in lung supernatant requires divalent ions but was not activated by carbamyl choline. During the past year, it was found that 2.5 mM $Ca^{++}$ increases the accumulation of cyclic-GMP 70-fold and increases that of cyclic-AMP 30-fold in lung cells. Moreover, a combination of carbamyl choline and 2.5 mM $Ca^{++}$ increases the accumulation of cyclic-AMP 70-fold, but partially inhibits the accumulation of cyclic-GMP. These findings are thus consistent with the view that carbamyl choline exerts its effects on cyclic nucleotide formation in part by modifying intracellular $Ca^{++}$ concentrations.

We have confirmed that succinylation of cyclic-GMP and cyclic-AMP increases the sensitivity of the immunoassay methods for these substances by 100-fold. With these sensitive methods, we have shown that cyclic-AMP in tracheal smooth muscle is increased by epinephrine and a vasoactive intestinal polypeptide and that theophylline potentiates these effects. We have also shown that cyclic-GMP in isolated pancreatic acinar cells is increased by cholinergic stimulants and that atropine blocks these effects.

Project No. Z01 HL 00801-01 LCP
1. Chemical Pharmacology
2. Enzyme-Drug Interaction
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Metabolic activation of α-methyldopa

Previous Serial Number: None

Principal Investigators: Dr. E. Dybing
Dr. J. R. Mitchell
Dr. S. D. Nelson
Dr. J. R. Gillette

Other Investigators: Mr. Kenneth Greene
Mr. John George

Cooperating Unit: Dr. Dybing is a Fogarty International Fellow.

Project Description:

Objectives: Renewed interest in the hepatic injury produced by methyldopa (MD) has been stimulated by recent reports that the antihypertensive drug may initiate chronic active liver disease, occasionally with a fatal outcome. The hepatic damage has been attributed to hypersensitivity rather than to direct toxicity, but careful review of the literature reveals that the syndrome is similar to that produced by isoniazid. Most individuals fail to show constitutional features indicative of an allergic response but usually demonstrate hepatic injury upon rechallenge only after lengthy re-exposure to MD. Moreover, MD produces mild, clinically covert, hepatic injury in 15% of recipients when liver function tests are monitored and thus the injury is not restricted to rare, idiosyncratic individuals. We have been interested in elucidating the role of the liver microsomal cytochrome P-450 system in a possible metabolic activation of MD.

Methods Employed: To assess the direct hepatotoxicity of MD, large doses (100-400 mg/kg) were administered i.p. or i.v. to animals. $^{3}$H-MD was incubated with rat or mice microsomal protein in the presence of a NADPH-generating system, and the covalent binding of reactive intermediates to microsomal proteins was determined at various substrate concentrations and under varying incubation conditions according to conventional methods.

Major Findings: MD produced mild hepatic injury with diffuse acidophilic bodies in male Fisher rats (min. toxic dose 250 mg/kg), but no increase in lipid diene conjugation nor depletion of glutathione were found. A large amount of covalent binding occurred when $^{3}$H-MD was incubated with rat or mice microsomal protein in the presence of NADPH and $O_2$ ($V_{max}$ 0.5 nmoles/mg/min in rats, 0.4 nmoles/mg/min in mice, $K_m$ 50 microM). The binding was

inhibited by a $CO:O_2$ atmosphere (9:1), indicating the involvement of cytochrome P-450. Moreover, antibody prepared against NADPH cytochrome c reductase inhibited binding by 49%. However, MD did not show P-450 binding spectra (Type I, II, or III) and its covalent binding was inhibited by superoxide dismutase, ascorbic acid (1 mM), ethylenediamine (20 mM) and glutathione (1 mM), indicating that activation by superoxide anion probably to the semiquinone radical was occurring. The covalent binding was inhibited by analogs such as l-dopa, dopamine, epinephrine, norepinephrine, catechol and resorcinol but not by 3-methoxy-α-methyl-tyrosine (3-O-methyldopa). These analogs, but not 3-O-methyldopa, also were found to covalently bind after microsomal activation. Additional studies with MD demonstrated that the rat microsomal system could be replaced by human hepatic microsomes or by a xanthine oxidase system and the binding again was inhibited by superoxide dismutase. Metabolic activation by superoxide anion may play a role in the toxicity of many catechols and catechol-like compounds.

Significance to Biomedical Research and the Program of the Institute: The results demonstrate that MD can be converted by hepatic cytochrome P-450 to a potent arylating agent. This reaction mechanism may be responsible for the hepatotoxicity of MD in patients.

Proposed Course of Project: Nearing completion; manuscripts are in preparation.

Keyword Description:

methyldopa
superoxide anion radicals
superoxide dismutase
xanthine oxidase

Honors and Awards: None

Publications: None

Project No. Z01 HL 00802-03 LCP
1. Chemical Pharmacology
2. Enzyme-Drug Interaction
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Effect of Spironolactone Analogues on Testicular P-450

Previous Serial No. NHLI-39, NHLI 191

Principal Investigators: Dr. H. Börner
Dr. J.R. Gillette

Other Investigator: Mr. John George

Cooperating Units: None

Project Description:

Objectives: In this laboratory it was shown that treatment with spironolactone causes a specific breakdown of testicular cytochrome P-450 in vitro. In order to elucidate the destruction mechanism, in vivo and in vitro studies with spironolactone and structural analogues were carried out.

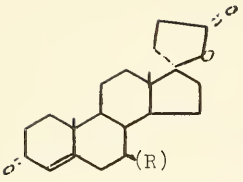
Methods Employed: Standard biochemical techniques were used.

Major Findings: In vitro studies with spironolactone revealed that spironolactone causes a breakdown of testicular cytochrome P-450 only when NADPH is present and the incubation is carried out at 37°C. The testicular cytochrome P-450 of the guinea pig was the most sensitive to spironolactone among rats, mice, rabbits and dogs.

Therefore, the effect of various analogues on the testicular cytochrome P-450 of guinea pig was checked in the presence of NADPH at 37°C.

Final concentrations of 14 to 540 µM were used. Here the limiting factor is the low solubility of the compounds. Propylene glycol was used to facilitate solution of spironolactone and analogues. However, propylene glycol caused destruction of cytochrome P-450 at concentrations higher than 300 µl/3 ml. Spironolactone dissolved in aqueous propylene glycol (100 µl/ml) causes a 50% destruction on testicular P-450 within 30 minutes in the presence of NADPH but not in the absence of NADPH. But all the other compounds decreased the P-450 by less than 10%. It seems likely that the initial hydrolysis of the thioacetyl-ester group which is assumed to be necessary before the formation of the reactive metabolite is hindered.

The studied compounds were:

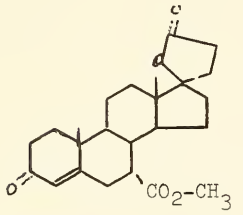



-R = S-C-$CH_3$ = Spironolactone

= --S-C-C(-$CH_3$)$_3$ = Sc 27825

= --S-C-$CH_2$-$CH_2$- = Sc 27937

= --S-C = Sc 27996

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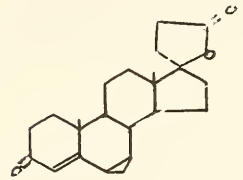

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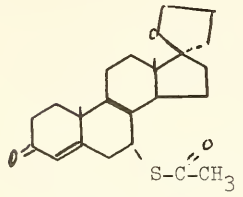
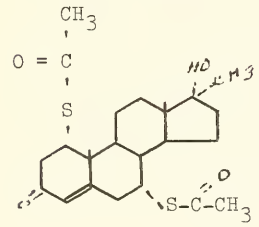



Spiroxazone




Emdabol

Significance to Biomedical Research and to the Program of the Institute: The elucidation of the mechanism by which spironolactone causes a breakdown of cytochrome P-450 may give information which could be helpful for the future design of diuretic steroids without side effects.

Proposed Course of Project: In vivo studies should be carried out for comparison.

Keyword Description: Spironolactone, Cytochrome P-450 and Testicular

Honors and Awards: None

Publications: None

Project No. Z01 HL 00803-06 LCP
1. Chemical Pharmacology
2. Enzyme-Drug Interaction
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The role of cytochrome $b_5$ in cytochrome P-450 systems

Previous Serial Number: NHLI 41

Principal Investigators: Dr. Henry A. Sasame
Dr. James R. Gillette

Other Investigators: None

Cooperating Unit: None

Project Description:

Objectives: Last year we reported the results of immunochemical studies which demonstrated unequivocally that the synergistic effect of NADH on NADPH-dependent metabolism of various compounds by cytochrome P-450 enzymes in liver microsomes is mediated by cytochrome $b_5$. In addition, we also demonstrated that cytochrome $b_5$ also plays a role in the NADPH-dependent metabolism of various compounds even in the absence of NADH. However, the anti-cytochrome $b_5$ antibody inhibited drug metabolism more than it inhibited NADPH oxidation. The objective of this project was therefore to determine the reason for this discrepancy.

Methods Employed: An anti-cytochrome $b_5$ antibody fraction was prepared from antiserum of sheep immunized against cytochrome $b_5$ purified from rat liver microsomes as reported in last year's report. The rate of superoxide anion formation by cytochrome P-450 enzymes in rat liver microsomes was assayed spectrophotometrically by following the rate of adrenochrome formation from epinephrine. The reduction rate of cytochrome $b_5$ in rat liver microsomes was measured in a stop-flow apparatus attached to Aminco DW-2 spectrophotometer. Standard biochemical procedures were used to measure other functional components of the microsomal cytochrome P-450 system.

Major Findings: 1) We have discovered that after the anti-cytochrome $b_5$ antibody blocks the transfer of electrons from cytochrome $b_5$ to the oxygenated cytochrome P-450 substrate complex, the concentration of the complex increases thereby increasing its rate of dissociation to oxidized cytochrome P-450 and superoxide anion ($O_2^{\cdot}-$). The latter was detected by measuring the cherry-red colored adrenochrome formed from epinephrine in the incubation media. As expected, the difference in the rate of adrenochrome formation in the presence and absence of the antibody gamma globin was greater when both NADH and NADPH were present than when only NADPH was present in the incubation media. In the absence of the antibody, NADH increases the rate of re-

duction of the complex formed in the presence of NADPH thereby decreasing the rate of formation of superoxide, whereas in the presence of the antibody, NADH scarcely alters the rate of superoxide formation. Since reduction of the oxygenated cytochrome P-450 complex is required for drug metabolism, the formation of superoxide accounts for the inhibition of drug metabolism without inhibition of NADPH oxidation.

2) Further evidence supporting the role of cytochrome $b_5$ as a source of second electron in NADPH-mediated cytochrome P-450 system in rat liver microsomes was unveiled by studies with liver microsomes isolated from rats pretreated with pregnenolone carbonitrile (PCN). Like the induction caused by phenobarbital, the induction by PCN increases both NADPH cytochrome c reductase and cytochrome P-450 levels in rat liver microsomes. However PCN markedly decreases the rate of cytochrome $b_5$ reduction and increases in the uncoupling between drug metabolism and NADPH oxidation. These conclusions were based on the following facts: i) The rates of cytochrome $b_5$ reduction by either NADH or NADPH were slower in liver microsomes from PCN treated rats than in those from phenobarbital treated rats. ii) The addition of ethylmorphine increased the rate of NADPH-mediated adrenochrome formation by liver microsomes to a greater extent after the PCN treatment than after the phenobarbital treatment. iii) The decrease in the steady state level of cytochrome $b_5$ caused by the addition of ethylmorphine was much greater in microsomes from PCN treated rats than in those from phenobarbital treated animals. The presence of the anticytochrome $b_5$ antibody decreased the steady-state level of reduced cytochrome $b_5$ in liver microsomes from PCN treated rats and prevented the decrease caused by ethylmorphine. Moreover, the addition of ethylmorphine lowered the NADH dependent steady state level of reduced cytochrome $b_5$ in liver microsomes from PCN treated animals but had no effect on the steady state level in microsomes from phenobarbital treated rats. iv) The anti-cytochrome $b_5$ antibody inhibited the NADPH dependent ethylmorphine metabolism in microsomes from PCN treated rats by as much as 50%, suggesting that PCN treatment also slows the reduction of the oxygenated cytochrome P-450 substrate complex by NADPH cytochrome c reductase as well as that by cytochrome $b_5$.

Significance to Biomedical Research and the Program of the Institute: Since it has been suggested that superoxide may cause cytotoxic effects, it is important to delineate the role of cytochrome $b_5$ in decreasing superoxide formation.

Proposed Course of Project: We shall study the effects of the anti-cytochrome $b_5$ antibody and PCN treatment on the in vitro metabolism of various drugs and steroids that cause toxicity in animal models.

Keyword Description:

cytochrome $b_5$
cytochrome P-450
superoxide

Honors and Awards: None

Publications:

Sasame, H.A., Thorgeirsson, S. S., Mitchell, J.R. and Gillette, J.R.: The role of cytochrome $b_5$ in cytochrome P-450 enzymes. In the Proceedings of the Second Philadelphia Conference on Heme Protein P-450. New York, Plenum Press, in press.

Project No. Z01 HL 00825-01 LCP
1. Chemical Pharmacology
2. Drug-Tissue Interaction
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Role of guanine nucleotides in vision

Previous Serial Number: None

Principal Investigator: Dr. G. Krishna

Other Investigators: Dr. N. Krishnan
Dr. G. Chader (NEI)

Cooperating Unit: Laboratory of Vision, NEI

Project Description:

Objectives: Retinal rod outer segment which is the photo receptor unit of the neuro retina contains extraordinarily high enzyme activities for the synthesis and degradation of cyclic GMP. Moreover, it contains very high protein kinase activity which specifically phosphorylates opsin. The role of these enzymes in vision is not clearly understood. The main objective of this study is to examine the effect of light exposure of the rod outer segments on the activities of these enzyme systems and to indicate the possible role of cyclic GMP in vision.

Methods Employed: Dark adapted bovine retinal rod outer segments were prepared by a method which did not involve homogenization. The rod outer segments were purified by sucrose gradient and were suspended in Tris hydrochloride buffer pH 7.6 containing 5 mM $Mgcl_2$ (1 mg protein/ml). The segments were exposed to light (100 ft candles) for specific periods of time. The assays of enzyme activities were carried out in the dark under diffused red light.

Guanylate cyclase and cyclic GMP phosphodiesterase were assayed according to the methods described in last year's report. GTP-opsin kinase or ATP-opsin kinase was measured using either 100 $\mu$M $\gamma$-$^{32}$P GTP or $\gamma$-$^{32}$P ATP. The phosphorylated protein were separated on a millipore filter. In some experiments the phosphorylated opsin was isolated on sepharose columns. Cyclic GMP was assayed according to the methods described in one of this year's reports (Frandsen and Krishna).

Major Findings: Bovine retinal rod outer segments contain the highest guanylate cyclase activity of any tissues thus far examined (5 nmoles of cyclic GMP formed per mg protein per minute). This enzyme undergoes light-induced inhibition (30%) within seconds. At the same time, the enzyme

responsible for degradation of cyclic GMP undergoes light-induced activation (10-fold) which requires the presence of ATP or GTP. Other nucleotides, ITP or UTP, have a smaller effect on the enzyme system while CTP has no effect.

Cyclic GMP is present in very high concentration in the rod outer segments (500 pmoles per mg protein) which is 200-300 times higher than any other tissues. The identity of cyclic GMP has been verified by three independent procedures including high pressure liquid chromatography. This nucleotide appears to be sequestered in the rod outer segments and does not undergo rapid degradation by the enzyme system present in the rod outer segments. The cyclic GMP also undergoes light-induced changes to a very small extent (20%).

Cyclic AMP is also present but only to the extent of 5 pmoles/mg protein.

The specific protein kinase present in the rod outer segments undergoes rapid activation (within one second) by light exposure of rod outer segments. This light-induced activation is mainly due to the conversion of rhodopsin to opsin by the light, and the enzyme phosphorylates only opsin as shown by chromatography on sepharose columns. The light activated opsin phosphorylation is effected by GTP or ATP. GTP appears to be more efficient and the phosphorylation by GTP is markedly inhibited by cyclic AMP and other adenine nucleotides. Inorganic phosphate also maredly inhibits GTP opsin kinase. The ATP opsin kinase is not inhibited to the same extent by adenine nucleotides and is maredly activated by phosphate. These differential effects as well as direct experimentation indicate that GTP opsin phosphorylation is not mediated through ATP.

Calcium, which is known to mimic light in its ability to hyperpolarize the membrane rod outer segments, markedly inhibits light-induced GTP opsin kinase. This indicates the effect of calcium in mimicking light may occur at the step beyond light-induced changes in enzyme activities.

Preliminary experiments indicate that the phosphorylated opsin molecule can transfer phosphate to ADP. The exact mechanism of ATP formation within the disc membrane is not clear, but the possibility of the involvement of phosphorylation of opsin in the transfer of phosphate from outside to inside the disc membrane is indicated, and this may result in the efflux of calcium from the disc involving calcium ATPase. The increase in calcium will block effectively the sodium channels resulting in hypopolarization and chus resulting in the conversion of light to electrical energy.

The above experiments strongly indicate the phosphorylatior of opsin by GTP and modulation by cyclic AMP and other adenine nucleotides may play an important role in vision.

Significance to Biomedical Research and the Program of the Institute: The finding that rod outer segments contain very high concentrations of cyclic GMP and the enzyme system for degradation and synthesis indicate that the photo receptor unit of the retina may represent a unique system where cyclic

GMP and guanine nucleotides play a more important role than the adenine nucleotides. This study will enable us to understand the role of cyclic GMP and GTP in other systems including the heart and lung in modulating cholinergic functions.

Proposed Course of Project: The role of GTP-opsin kinase in the transfer of phosphate from outside of the disc membrane to inside and the role of calcium in mimicking light will be investigated in detail.

Keyword Description:

cyclic GMP
guanine nucleotides
opsin phosphorylation
retinal rod outer segments
vision

Honors and Awards: None

Publications:

Chader, Gerald J., Fletcher, R.T., and Krishna G.: Light-induced phosphorylation of rod outer segments by guanosine triphosphate. Biochem. & Biophys. Research Comm., in press.

Project No. Z01 HL 00826-02 LCP
1. Chemical Pharmacology
2. Drug-Tissue Interaction
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies on the Covalent Binding of Chloramphenicol

Previous Serial No. NHLI-52

Principal Investigators: Dr. Lance R. Pohl
Dr. B.G. Reddy
Dr. Gopal Krishna

Other Investigator: Ms. Ethel Boykins

Cooperating Unit: None

Project Description:

Objectives: Chloramphenicol ($^{14}C$) has previously been shown to be covalently bound in vivo predominantly to proteins of the liver, bone marrow, and plasma. The present research is being conducted in order to determine the mechanism(s) by which chloramphenicol is activated to metabolites that react with tissue macromolecules. This study will hopefully lead to a better general understanding of the mechanism(s) involved in chloramphenicol-induced bone marrow damage. In addition, it may also lead to an intimate understanding of the chemical reactions between reactive metabolites and biological molecules. Such interactions appear to be involved in the hepatotoxicity, and carcinogenicity induced by several chemical agents. Similar interactions may also be involved in the development of blood dyscrasias, such as chloramphenicol-induced asplastic anemia.

Methods Employed: 1) $^{3}H$-Chloramphenicol (Fig.1) was synthesized by reaction of the keto-derivative of chloramphenicol with $^{3}H$-calcium borohydride. The keto derivative of chloramphenicol was prepared by oxidation of chloramphenicol with N-bromosuccinamide.

2) Covalent binding of chloramphenicol was studied in vitro utilizing rat liver microsomes and NADPH as described in earlier reports. For these studies double-labeled ($^{14}C$ and $^{3}H$) chloramphenicol was employed.

3) In vivo covalent binding to bone marrow and other tissues of the rat was studied by injecting double-labeled ($^{14}C$ and $^{3}H$) chloramphenicol (Fig.1) (30 mg/kg, po) to phenobarbital-pretreated rats. Various tissues were removed at the end of 24 hours and covalent binding to various tissues was studied as reported earlier.

Major Findings: 1) Covalent Binding in vitro. Both $^{3}H$ and $^{14}C$ chloramphenicol bind to rat liver microsomes at the rate of 200 pmoles/mg protein. The rate of the reaction appears to slow after 4 minutes of incubation. After 10 minutes about 20% more $^{3}H$ than $^{14}C$ chloramphenicol is bound covalently to rat liver microsomes. These results indicate that the majority of the covalently bound molecules contain the entire molecule of chloramphenicol. The most likely bioactivation of chloramphenicol that could lead to covalent binding appears to be arene oxide formation, hydroxylation and/or free radical formation at the dichloroacetamide group.

2) Covalent Binding in vivo. When double labeled chloramphenicol was administered to rats at a dose of 30 mg/kg po, both $^{3}H$ and $^{14}C$ appear to be bound covalently to various tissue macromolecules. The covalent binding of $^{14}C$ chloramphenicol appears to be similar to that obtained in last year's studies. However, the binding of $^{3}H$ chloramphenicol is markedly different in various tissues. Liver contains about 30-40% of $^{3}H$ chloramphenicol bound covalently as compared to $^{14}C$ chloramphenicol while blood and bone marrow contain only 10% of $^{3}H$ chloramphenicol as compared to $^{14}C$ chloramphenicol. Thus, it appears that in vivo chloramphenicol may undergo cleavage of the dichloroacetamide group either before or after binding to macromolecules resulting in differential binding of $^{14}C$ and $^{3}H$ chloramphenicol. It is also conceivable that bioactivation may involve an oxidation of chloramphenicol keto compound resulting in the loss of $^{3}H$ before covalent binding.

Significance of Biomedical Research and the Program of the Institute: The finding that the covalent binding of $^{14}C$ and $^{3}H$ chloramphenicol is markedly different in vivo in comparison to in vitro indicates a more complex nature of bioactivation of drug molecules in vivo which may be responsible for the drug-induced tissue damage. Thus it is not possible to predict any mechanism of drug activation from studies utilizing liver microsomes in vitro alone.

Proposed Course of Project: Various tissue macromolecules containing covalently bound $^{3}H$ and $^{14}C$ chloramphenicol will be hydrolyzed with pronase in order to isolate the amino acids containing the label from the chloramphenicol molecules. The analysis of the material would enable us to arrive at a mechanism of activation and covalent binding of chloramphenicol.

We also propose to study the suceptability of various animal species to chloramphenicol-induced bone marrow damage. Since a number of possible metabolites of chloramphenicol, such as dichloracetic acid, chloramphenicol base and keto derivative of chloramphenicol, are available in sufficient amounts we propose to study whether these metabolites are covalently bound and are capable of producing bone marrow damage in various animal species.

Since numerous attempts in this laboratory and others have failed to induce aplastic anemia in experimental animals with chloramphenicol, we propose to determine whether chloramphenicol causes aplastic anemia in animals whose hemopoietic system is markedly stimulated by pretreatment with phenylhydrazine.

Keyword Description: Covalent Binding and Chloramphenicol

Honors and Awards: None

Publications:

Krishna, G. and Bonanomi, L.: Covalent Binding of Chloramphenicol as a Biochemical Basis for Chloramphenicol-induced Bone Marrow Damage. In Morselli, P.L., Garattini, S. and Cohen, S.N. (Eds.): Drug Interactions. New York, Raven Press, 1974, pp. 173-180.

Krishna, G.: Covalent binding of drugs to tissue macromolecules as a biochemical mechanism of drug toxicities with special emphasis on chloramphenicol and thiamphenicol. Postgraduate Medical Journal 50: 73-77, 1974.

Rao, G.S., Krishna, G. and Gillette, J.R.: The enzymatic formation of chemically reactive metabolites of N-nitroso-monomethyl tripelennamine by a mechanism other than N-dealkylation. Biochemical Pharmacology, in press.

Asghar, K., Reddy, B.G. and Krishna, G.: Histochemical localization of glutathione in tissues. The Journal of Histochemistry and Cytochemistry, in press.

Docks, E.L. and Krishna, G.: Covalent binding of trans-stilbene to rat liver microsomes. Biochemical Pharmacology, in press.

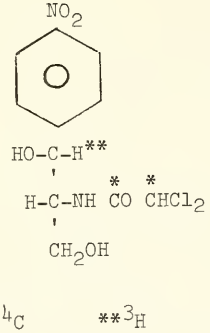



Fig. 1 CHLORAMPHENICOL

Project No. Z01 HL 00827-01 LCP
1. Chemical Pharmacology
2. Drug-Tissue Interaction
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Role of intestinal flora on nitrobenzene-induced toxicities.

Previous Serial Number: None

Principal Investigators: Dr. B. G. Reddy
Dr. Lance R. Pohl
Dr. Gopal Krishna

Other Investigators: None

Cooperating Unit: None

Project Description:

Objectives: Nitrobenzene has been known to have a toxic effect upon the hemopoetic system. One of the major pathological changes observed with nitrobenzene administration in acute studies is methemoglobinemia. In order to understand the mechanism of formation of methemoglobin, we have studied the relationship of biotransformation of nitrobenzene to the toxicity. This investigation will further serve as a model study for more complex molecules. In particular, these studies may serve as an important model for studying various toxicities produced by the antibiotic chloramphenicol which contains a p-nitrophenyl group.

Methods Employed: Male, Sprague Dawley rats, 180-220 g, from Hormone Assay Labs were used in this study. Nitrobenzene (200 mg/kg) and m-dinitrobenzene (20 mg/kg) were administered intraperitoneally to rats, and the rate of formation and disappearance of methemoglobin was measured. In order to understand the role, if any, of the cytochrome P-450 systems in these reactions, the experiments have also been conducted with phenobarbital and piperonyl butoxide pretreated rats since these two agents are known to induce and inhibit, respectively, these systems. Similar experiments have also been conducted in rats whose intestinal flora was destroyed by antibiotic treatment and in germ-free rats. These studies were run in order to assess the involvement of the intestinal flora in the biotransformation and toxicity of nitrobenzene.

Major Findings: As shown in Table 1, route of administration had a significant ($P < .01$) effect on the level of methemoglobin formation by both nitrobenzene and meta-dinitrobenzene. The effect on methemoglobin formation was more pronounced when nitrobenzene was administered intraperitoneally in comparison to the oral route. When nitrobenzene was administered to rats by oral route, the methemoglobin levels never reached above 6% of total

hemoglobin during the 8 hr period whereas, administration of nitrobenzene by intraperitoneal route, the methemoglobin levels reached a peak within 2 hr and then declined. It is evident from the table, the route of administration had a small but significant effect ($P < .01$) on the methemoglobin formation induced by meta-dinitrobenzene. Based on these observations, the intraperitoneal route was chosen for further studies. The highest levels of methemoglobin in both i.p. and oral route was found to be between 1 and 2 hr after administration of these compounds.

The effects of phenobarbital and piperonyl butoxide pretreatments on methemoglobin formation are shown in Table 2. Table 2(a) shows that phenobarbital increased significantly ($P < .05$) methemoglobin formation by nitrobenzene compared to normal rats. This suggests that the liver may have a role in the bioactivation of nitrobenzenes to a metabolite which is responsible for methemoglobin formation. Piperonyl butoxide pretreatment did not significantly affect methemoglobin formation by nitrobenzene. However, the rate of methemoglobin disappearance appears to be similar in all cases.

With m-dinitrobenzene, the peak levels of methemoglobinemia seems to be affected by the pretreatment with phenobarbital and piperonyl butoxide [Table 2(b)]. However, in this case, pretreatment with piperonyl butoxide and phenobarbital tend to decrease the level of methemoglobinemia produced by m-dinitrobenzene. More work is needed before these differences in the behavior of these nitrobenzenes can be explained.

Table 3(a) shows the effect of removal of intestinal flora from the rat on nitrobenzene-induced methemoglobin formation. No methemoglobin was formed by administration of nitrobenzene to germ free rats, whereas the same rats, after being acclimatized for a week in the animal room, responded to nitrobenzene with formation comparable to normal. Similar results were also obtained in the rats whose intestinal flora was removed by antibiotic treatment (neomycin, bactrin and tetracycline).

In the case of m-dinitrobenzene [Table 3(b)], it appears the germ free condition also has an effect on methemoglobin formation. However, the apparent effect of the intestinal flora on the methemoglobin formation by m-dinibrobenzene is considerably lower than nitrobenzene. The methemoglobin level for the germ free and antibiotic treated rats started low compared to corresponding normals and reached the peak between 2 to 3 hr and then declined.

Significance to Biomedical Research and the Program of the Institute: The finding that the intestinal flora markedly influence the toxicity produced by nitrobenzene adds another environmental factor in which the enzyme system of the bacteria in the intestine may play a crucial role in certain drug-induced diseases.

Proposed Course of Project: In the light of the available data from the germ-free and antibiotic-treated rats, we are conducting experiments to determine the relative contribution of enzyme systems in liver, intestinal

musoca and intestinal bacteria to the formation of nitrobenzene-induced methemoglobinemia. (1) A series of studies are being conducted in rats whose bile flow has been interrupted in order to assess the importance of liver in the formation of methemoglobin by nitrobenzene. These studies will also enable us to assess the role of bacterial as well as intestinal mucosal enzyme system in the formation of the metabolite responsible for the methemoglobin formation by nitrobenzene. (2) We also shall attempt to identify the active metabolite(s) of nitrobenzene responsible for methemoglobin formation by comparing the spectrum of the biotransformation products in germ-free and acclimatized rats.

It is hoped that this research will lead to a better understanding and serve as a model study for the assessment of the relative importance of the intestinal flora in the metabolism and toxicity of drugs and environmental chemicals. In particular, this study may have relevance to the hemapoetic toxic properties of chloramphenicol, which contains a p-nitrophenyl ring in its structure. In addition, the identification of the active metabolites of nitrobenzene, should help elucidate the general mechanism for the formation of methemoglobin.

Keyword Description:

| | |
|---|---|
| germ-free rats | nitrobenzene |
| intestinal flora | phenobarbital |
| methemoglobinemia | piperonyl butoxide |

Honors and Awards: None

Publications: None

Table 1

Effect of route of administration on methemoglobin formation

| Time (hr) | Nitrobenzene (200 mg/kg) | | m-Dinitrobenzene (20 mg/kg) | |
|---|---|---|---|---|
| | Oral | Intraperitoneal | Oral | Intraperitoneal |
| | per cent methemoglobin | | per cent methemoglobin | |
| 0.5 | 0.8 ± 0.8 | 12.3 ± 9.7 | 36.3 ± 3.3 | 47.7 ± 2.4* |
| 1 | 1.2 ± 1.2 | 27.0 ± 6.6* | 45.0 ± 3.0 | 52.4 ± 1.3 |
| 2 | 6.1 ± 4.3 | 33.4 ± 8.4* | 44.4 ± 3.2 | 44.5 ± 1.5 |
| 4 | 3.5 ± 2.2 | 23.0 ± 10.0* | 21.5 ± 4.3 | 21.0 ± 2.4 |
| 8 | 0.3 ± 0.3 | 10.7 ± 6.9 | 4.7 ± 2.4 | 0 ± 0 |

The data are expressed as means ± S.E. (N = 3).

*$P < .05$

Table 2(a)

Effect of phenobarbital and piperonyl butoxide pretreatment on nitrobenzene (200 mg/kg ip)-induced methemoglobin formation

| Time | Controls | Phenobarbital treated | Piperonyl butoxide treated |
|---|---|---|---|
| | | per cent methemoglobin | |
| 1 hr | 29.0 ± 2.7 | 34.0 ± 4.7* | 25.3 ± 3.5 |
| 2 hr | 29.7 ± 2.6 | 32.7 ± 3.8 | 29.2 ± 3.9 |
| 4 hr | 22.9 ± 2.8 | 31.6 ± 3.3 | 28.1 ± 2.9 |
| 6 hr | 21.0 ± 1.4 | 25.6 ± 2.1 | 31.5 ± 0.8 |
| 8 hr | 15.9 ± 3.3 | - | 19.1 ± 2.0 |

The data are expressed as means ± SE (N = 6; phenobarbital-pretreated N = 11).

*$p < 0.05$

Table 2(b)

Effect of phenobarbital or piperonyl butoxide pretreatment on m-dinitrobenzene (20 mg/kg i.p.) induced methemoglobin formation.

| Time interval | Control | Phenobarbital pretreatment | Piperonyl butoxide pretreatment |
|---|---|---|---|
| | | per cent methemoglobin | |
| 1 hr | 44.9 ± 4.5 | 40.9 ± 5.2 | 39.7 ± 3.3 |
| 2 hr | 38.4 ± 5.8 | 36.6 ± 8.3 | 37.5 ± 4.1 |
| 4 hr | 18.2 ± 3.9 | 9.1 ± 2.6 | 16.7 ± 3.4 |
| 6 hr | 9.8 ± 5.6 | 0.8 ± 0.5 | 5.4 ± 2.0 |
| 8 hr | 1.4 ± 1.4 | 0.1 ± 0.1 | 0 ± 0 |

The data are expressed as means ± SE (N = 6).

Table 3(a)

Nitrobenzene (200 mg/kg i.p.)-induced methemoglobin formation in germ free, germ free-acclimatized and antibiotic-pretreated rats.

| Time (hr) | Germ free rats | Germ free acclimatized rats | Antibiotic pretreated rats |
|---|---|---|---|
| | | per cent methemoglobin | |
| 1 | 0 | 37.5 ± 4.2 | 0 |
| 2 | 0 | 37.8 ± 3.4 | 0 |
| 4 | 0 | 26.1 ± 3.4 | 0 |
| 5 | 0 | 5.0 ± 0.5 | 0 |
| 7 | 0 | 4.4 ± 0.7 | 0 |

Table 3(b)

m-Dinitrobenzene (20 mg/kg i.p.)-induced methemoglobin formation in germ free, germ free-acclimatized and antibiotic-pretreated rats.

| Time (hr) | Germ free rats | Germ free acclimatized rats | Antibiotic pretreated rats |
|---|---|---|---|
| | | per cent methemoglobin | |
| 1 | 6.9 ± 0.8 | 42.7 ± 4.2 | 18.7 ± 1.1 |
| 2 | 22.6 ± 0.7 | 26.9 ± 2.9 | 23.8 ± 3.2 |
| 4 | 28.9 ± 2.5 | 4.96 ± 2.9 | 20.6 ± 0.6 |
| 5 | 24.5 ± 3.7 | 0 | 17.8 ± 1.3 |
| 7 | 11.0 ± 6.4 | 0 | 4.5 ± 2.1 |

The data are expressed as means ± SE (N = 3; antibiotic-pretreated N = 6).

Project No. Z01 HL 00828-01 LCP
1. Chemical Pharmacology
2. Drug-Tissue Interaction
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Role of lung cyclic nucleotides in paraquat toxicity

Previous Serial Number: None

Principal Investigators: Dr. N. Krishnan
Dr. G. Krishna

Other Investigators: None

Cooperating Unit: None

Project Description:

Objectives: Paraquat is widely used as an herbicide. The plants are destroyed when the leaf surfaces are exposed to paraquat and the herbicide is inactivated by absorption onto clay minerals. The paraquat toxicity in human has been well documented, the pulmonary tissue being the main target for paraquat toxicity. Toxicity in man occurs mainly after drinking solutions of these compounds or after inhalation of dust. Death mainly occurs after 2 to 5 days with pulmonary edema and congestion with hyaline membrane formation. Some animal species become hyperexcitable after doses and have convulsions after lethal doses. In view of the acute toxicity of this drug, attempts are being made in several laboratories to gain an insight into the mode of toxicity of this compound on the lung. The only information that is presently available is that paraquat appears to be transported by an energy dependent process and stored within the lung. The stored paraquat in lung tissue appears to not be metabolized and slowly excreted as such in urine, and thus accounts for all the toxic effects observed in the lungs. Various attempts are being made to understand the types of lung cells involved in the paraquat toxicity. The cells of the lung which has the highest capacity to take up and bind paraquat would be the likely target for paraquat toxicity. Since paraquat produces pulmonary edema and since cyclic AMP has been known to be involved in movement of ions across various membranes, we have studied the effect of paraquat on the levels of cyclic AMP in the lung.

Methods Employed: Paraquat (25 mg/kg) was administered intraperitoneally to male Sprague-Dawley rats (150 g). Rats were sacrificed at 15 min and 30 min intervals after administration of paraquat and the lung tissue was removed rapidly and frozen in liquid nitrogen. The cyclic AMP and cyclic GMP were extracted with 10% perchloric acid in 50% methanol. Cyclic AMP and cyclic GMP were isolated by Dowex 1 chromatography and were measured using specific radioimmunoassays.

Major Findings: Lung cyclic AMP levels were decreased by 70% over the control 15 min after administration of paraquat; while 30 min after administration of paraquat, cyclic AMP levels were decreased only by 30%. There were no changes in lung cyclic GMP levels at these times.

Significance to Biomedical Research and to the Program of the Institute: A marked reduction in lung cyclic AMP levels within 15 min after administration of paraquat suggests that the cyclic AMP system is grossly affected and may reflect as one of the early changes that occurs in the onset of toxicity in the lung. Further studies correlating paraquat-induced changes in cyclic AMP levels in the body with its toxicity are essential to elucidate this point.

Proposed Course of Project: We propose that studies on various neurohormonal regulation of paraquat-induced changes in cyclic AMP in the lung may help to explain if cyclic AMP is involved in paraquat-induced tissue damage. Improved techniques that are being available for the isolation of Type II alveolar epithelial cells will greatly help to understand if these cells are capable of concentrating paraquat and thus involved in paraquat-induced toxicity.

Keyword Description:

| | |
|---|---|
| cyclic AMP | lung |
| cyclic GMP | paraquat |
| cyclic nucleotides | Type II alveolar epithelial cells |

Honors and Awards: None

Publications: None

Project No. Z01 HL 00829-01 LCP
1. Chemical Pharmacology
2. Drug Tissue Interaction
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Present Title: Effects of Ca++ and Carbachol on cGMP in Lung Cells

Previous Serial No.: None

Principal Investigators: Dr. N. Krishnan
Dr. G. Krishna

Other Investigators: None

Cooperating Unit: None

Project Description:

Objectives: Most studies of lung function have been carried out with either whole lung or lung slices. Because lung contains at least 40 different types of cells, such studies are difficult to interpret. Clearly preparations are needed that are predominantly of one single cell type. However, as a first step, studies with preparations consisting of mixed population of lung cells are needed to evaluate various techniques for separating and isolating functional cell types. Our initial studies on the effect of cholinergic agents on cyclic nucleotide levels in lung cells were carried out on viable lung cells prepared by proteolysis of the lung tissue according to the method of Gould et al. (Science 178:1209, 1972).

Methods Employed: Male Sprague Dawley rats weighing 150 g were anesthetized, the lungs were artificially ventilated and perfused at 38°C in situ with Krebs-Ringer phosphate buffer (Ca++ and Mg++ free), containing glucose and albumin. The lung tissue, after removal, was freed of extra pulmonary bronchi and connnective tissues and sliced to 1 mm cubes in a tissue slicer and incubated in Ca++-Mg++ free KRP albumin buffer containing 1 mg of crude collageanse, 2 mg of pronase, 0.5 mg chymopapain, 10 units of elastase, 0.03 mg deoxyribonuclease and 0.005 ml of crude elastase per ml of buffer. The lung tissue obtained from 5 rats was incubated with the enzyme mixture (30 ml) at 38°C for 45 minutes with gentle agitation on a Dubnoff Shaker. After incubation, the contents were passed through a silk cloth to remove cell debris and connective tissues. The filtrate was centrifuged for 15 seconds at 3,000 RPM and the cells were washed twice with Krebs-Ringer phosphate-albumin buffer and diluted with the same buffer and used for the experiments. A small aliquot of the lung cells was fixed with 2% glutaraldehyde for electron microscopic examination.

Our studies on the effect of calcium and carbamylcholine on cyclic nucleotide levels were carried out by incubating the lung cells for 2 minutes at 38°C. The reaction was terminated by addition of 10% perchloric acid. Cyclic AMP and cyclic GMP were separated by chromatography on Dowex-1 formate columns and assayed by the radioimmunoassay method of Steiner (J. Biol. Chem. 247:1106, 1972).

Major Findings: Electron microscopic examination of lung cells showed that about 50% of the cells obtained are Type II alveolar epithelial cells (Type II pneumocytes). The rest of the cells consist mainly of macrophages, clara cells, lymphocytes and Type I epithelial cells.

Last year, we reported that carbamylcholine did not activate lung supernatant guanylate cyclase; but that the enzyme required divalent cations. During the past year we tested the effect of $Ca^{++}$ on cyclic GMP accumulation in lung cells. At a concentration of 2.5 mM $Ca^{++}$ stimulated cyclic GMP accumulation over the basal levels by seventy-fold (Basal level, 0.4 pmoles; $Ca^{++}$ stimulated levels 28 pmoles/ml lung cells). Under these conditions, however, the addition of carbamylcholine partially prevented the stimulatory effect of $Ca^{++}$ on the accumulation of cyclic GMP.

We also measured the cyclic AMP levels in the same lung cell samples. $Ca^{++}$ (2.5 mM) stimulated cyclic AMP accumulation somewhat less markedly to about 30-fold over the basal levels (0.4 pmole/ml cells basal; 13 pmoles $Ca^{++}$ stimulated). Addition of carbamylcholine together with calcium resulted in further stimulation of cyclic AMP formation to about 70-fold (28 pmoles/ml cells).

These results point to the crucial role played by $Ca^{++}$ in regulating cyclic nucleotide levels within lung cells. Further experiments are in progress to elucidate the mechanism of these effects of $Ca^{++}$ and hormone.

Significance to Biomedical Research and the Program of the Institute: A study on the effect of carbamylcholine in the lung may help to undersstand the role of cyclic nucleotides in the cholinergic action in this organ. The profound effect of $Ca^{++}$ observed in our studies will help to understand the role of divalent metal ion in hormone modulation of lung cell functions.

Proposed Course of Project: Attempts will be made to isolate the Type II alveolar epithelial cells (Type II pneumocytes) from other cells at least up to 90% of one cell type; these are the cells that synthesize surfacants which are essential in maintaining the structural integrity of the pulmonary tissue. The possible role of cyclic AMP and cyclic GMP in the surfactant formation and the involvement of cholinergic and adrenergic systems in the surfactant synthesis will be investigated in Type II cells of the lung in order to elucidate the regulatory role played by calcium. Studies will also be carried out to understand the nature of receptor(s) involved in carbamylcholine and calcium mediated stimulation of cyclic AMP formation. Moreover, these cells will be utilized to study paraquat-induced changes in cyclic

AMP levels in the lung and to elucidate cell type of the lung that has the capacity to concentrate paraquat.

Keyword Description: Lung cells, calcium, carbachol and cyclic GMP

Honors and Awards: None

Publication:

Rodbell, M. and Krishna, G.: Preparation of Isolated Fat Cells and Fat Cell "Ghosts"; Methods for Assaying Adenylate Cyclase Activity and Levels of Cyclic AMP. In Fleischer, S. and Packer, L. (Eds.): Methods in Enzymology. New York, Academic Press, 1974, Vol. XXXI, Part A, pp. 103-114.

Project No. Z01 HL 00830-01 LCP
1. Chemical Pharmacology
2. Drug Tissue Interaction
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Role of Cyclic Nucleotides in Hormone Action

Previous Serial No.: None

Principal Investigators: Dr. Erik K. Frandsen
Dr. Gopal Krishna

Other Investigator: Mrs. C. McDaniels

Cooperating Units: None

Project Description:

Objectives: The role of cyclic AMP as a mediator of a variety of hormones is well documented but the role of cyclic GMP is not yet clearly understood. So far the only system that may be mediated by cyclic GMP, appears to be the cholinergic (muscarinic) system. One of the main problems involved in the study of the role of cyclic GMP in hormone action appears to be the transient effect that the hormone produces on the cyclic GMP system and the magnitude of the effect. Most of the tissues appear to contain only 1/100 of cyclic GMP levels as compared to cyclic AMP levels. Moreover, only a small portion of cells in the tissue respond to hormone stimuli and thereby restricting the magnitude of overall response.

We have attempted to solve these problems by developing a sensitive method for assay of cyclic GMP and modifying the methods available for cell isolation in order to get a population of cells which are enriched with one type of cell from tissues which consist of numerous cell types.

Methods Employed: Cyclic AMP and cyclic GMP were extracted with perchloric acid from tissues or cells and separated on Dowex 1 formate (BioRad A.G. 1 x 4 200-400 mesh) column. Cyclic AMP and cyclic GMP were eluted with 2N and 4N formic acid, respectively. Aliquots of cyclic AMP and cyclic GMP fractions were lyophilized, dissolved in 50 µl of water and were succinylated with 5 µl of a mixture containing 1 ml of succinic anhydride in acetone (200 mg/ml) and 0.36 ml of triethylamine. After 10 minutes at room temperature, the reaction was terminated by addition of 1 ml of sodium acetate buffer (0.05 M, pH 6.2) and a 200 µl aliquot was taken for radioimmunoassay. Standards containing cyclic GMP were run with the samples. The radioimmunoassay was performed at 4°C overnight (12-16 hr) after addition of 50 µl of $^{125}$I-antigen and 50 µl of cyclic GMP antibody (1:25000). The free $^{125}$I-antigen was separated from the bound by precipitation with cold ethanol (80%

final concentration). Bovine serum albumin (0.5 - 1 mg) was added to aid a complete precipitation of the bound antigen. The unbound radioactivity was determined in a scintillation counter.

Pancreatic acinar cells were isolated with collagenase treatment.

Major Findings: The succinylation of cyclic GMP by succinic anhydride requires the presence of triethyl amine; very little succinylation occurs in the absence to triethylamine. Table 1 shows the dgree of succinylation with various combinations of succinic anhydride and triethylamine. The maximum succinylation occurs with 10 mg of succinic anhydride in the presence of 20 µl of triethylamine.

Table 2 shows that succinylation of cyclic GMP, by a mixture containing succinic anhydride and triethylamine is independent of cyclic GMP concentration. Almost quantitive succinylation is obtained using 30 µl of mixture containing 1 ml of succinic anhydride (200 mg/ml in acetone) and 0.36 ml of triethylamine.

The succinylation occurs virtually instantaneously when carried out in aqueous medium, but much more slowly when carried out in a nonaqueous medium like acetone.

The succinylated product may be isolated from $^{3}$H-cyclic GMP by thin layer chromatography on silica gel (butanol:acetic acid:water 4:1:2).

It has been identified as 2'-O-cyclic GMP by a variety of techniques including high pressure liquid chromatography. Moreover, the succinylated material can be hydrolyzed to cyclic GMP.

The affinity of the cyclic GMP antibody for the succinylated cyclic GMP was found to be 100 times higher than for cyclic GMP. Thus, succinylation increases the detectability of cyclic GMP 100-fold by radioimmunoassay. Now it is possible to quantitate 2 fentomoles of cyclic GMP.

Ethanol at concentrations higher than 80% efficiently precipitates the bound antigen. Very little dissociation of antigen-antibody complex occurs in ethanol provided the mixture is kept cold (4°C).

A similar method has also been developed for assay of cyclic AMP. The degree of succinylation as well as increase in affinity of the succinylated cyclic AMP to cyclic AMP antibody appears to be very similar to that obtained for cyclic GMP. The method has been applied for the study of the role of cyclic nucleotide in catecholamine and vasoactive intestinal polypeptide (VIP) induced relaxation in the tracheal smooth muscle. Preliminary studies indicate that cyclic AMP is increased when tracheal smooth muscles are exposed to either epinephrine or VIP. Theophylline which has been known to potentiate the relaxation of the tracheal smooth muscle produced by these hormones also potentiates the increase in cyclic AMP. Cyclic GMP levels

were not altered by these hormones.

The method for assay of cyclic nucleotides also has been utilized in the study of the role of cyclic GMP in mediating cholinergic response in isolated pancreatic acinar cells. Preliminary results indicate that the cholinergic stimulant carbamyl choline, rapidly increase cyclic GMP levels thereby indicating that cyclic GMP may be involved in the cholinergically mediated pancreatic acinar cell functions.

Significance to Biomedical Research and to the Program of the Institute: The development of a simple and sensitive assay of cyclic nucleotides in the fentomole range should greatly help in the understanding of the role of cyclic nucleotides in various hormone actions. With the advent of development of simple methods for isolation of cells of single cell type for various tissues like liver, lung, pancreas, will greatly help in the understanding of the role of these cells and how they are modulated by various hormones.

Proposed Course of Project: The role of cyclic nucleotides in mediating sympathetic, histaminic and cholinergic functions in tracheal smooth muscles will be studied in greater detail. The role of cyclic GMP in mediating cholinergic functions in pancreatic acinar cells will be studied in order to understand the mechanism by which cholinergic agents activate guanylate cyclase in the cells.

Parasympathetic nerve endings have been identified in the islets of Langerhans in the pancreas. Stimulation of these nerves causes increased secretion of insulin which is blocked by atropine. Since in other systems cyclic GMP has been shown to mediate cholinergic function, the role of cyclic GMP in insulin secretion will be studied.

Keyword Description: Cyclic AMP, Cyclic GMP, Assay System and Isolated Cells

Honors and Awards: None

Publications: None

Table 1

Effect of increasing succinic anhydride and triethylamine on the succinylation of cGMP

| µl triethylamine | mg succinic anhydride | | | |
|---|---|---|---|---|
| | 1 | 3 | 4 | 10 |
| | per cent succinylation | | | |
| 0 | 1.8 | 1.9 | 1.9 | 2.4 |
| 0.5 | 13.5 | 11.0 | 7.5 | 8.8 |
| 1 | 52.9 | 33.3 | 20.2 | 14.9 |
| 2 | 71.0 | 67.9 | 52.0 | 26.7 |
| 5 | 66.2 | 90.4 | 94.0 | 78.0 |
| 10 | 45.3 | 73.0 | 95.4 | 97.0 |
| 20 | 44.7 | 60.0 | 86.5 | 96.5 |

$^{3}$H-cGMP (1 pmole - 5000 cpm) was dissolved in 100 µl water. Succinic anhydride was added in 20 µl acetone followed triethylamine. The mixture was incubated at room temperature for 30 min. Aliquots were chromatographed on silica gel and developed with butanol:acetic acid:water (4:1:2). The cGMP and succinylated cGMP spots were removed and radioactivity was determined.

Table 2

Effect of increasing cGMP on the succinylation of cGMP

| cGMP pmoles | µl of succinylation reagents* | | |
|---|---|---|---|
| | 5 | 10 | 30 |
| | per cent succinylation | | |
| 0 | 51.0 | 66.7 | 94.6 |
| 1 | 46.2 | 63.7 | 95.1 |
| 10 | 50.0 | 64.0 | 94.7 |
| 100 | 44.6 | 63.8 | 96.1 |
| 1,000 | 50.0 | 66.0 | 95.4 |
| 10,000 | 47.3 | 68.8 | 95.0 |
| 100,000 | 47.1 | 67.1 | 94.7 |

*200 mg succinic anhydride in 1 ml acetone and 0.36 ml triethylamine

$^{3}$H-cGMP (1 pmole - 5000 cpm) was dissolved in 100 µl of aqueous solutions of various concentrations of cGMP. cGMP was succinylated by adding a mixture containing succinic anhydride and triethylamine (200 mg succinic anhydride in 1 ml of acetone plus 0.36 ml triethylamine). cGMP and succinylated cGMP were separated and determined as described under Table 1.

Project No. Z01 HL 00851-02 LCP
1. Chemical Pharmacology
2. Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Paraquat toxicity in rat lung.

Previous Serial Number: NHLI-46

Principal Investigators: Dr. Harriet M. Maling
Dr. Elise Ann Brandenburger Brown
Dr. James R. Gillette

Other Investigators: Mrs. Martha A. Williams
Mr. Wilford Saul

Cooperating Unit: Dr. Brown is associated with the Pulmonary Branch, NHLI.

Project Description:

Objectives: In this project, we hope to elucidate the mechanisms responsible for the pulmonary toxicity of paraquat, 1,1'-dimethyl-4,4'-dipyridylium dichloride, an herbicide which is extensively used in agriculture. Demonstration of a treatment for paraquat poisoning in rats should have clinical applications in the treatment of accidental paraquat poisonings in man. An understanding of the mechanisms involved in the pulmonary toxicity of paraquat should give insight into various mechanisms responsible for the development of pulmonary edema after exposure to other agents.

Methods Employed: Data has been collected on the influence of various treatments on the 48-hr mortality of paraquat, injected i.p. Lung weights and gross pathologic grading, as defined in the Annual Report for 1973-74, were used as indicators of the extent of pulmonary edema.

Diene conjugation of lung microsomal lipids was measured by a modification of the method described by Rao and Recknagel (Exp. Mol. Path. 9: 271, 1968) for measuring diene conjugation of liver microsomal lipids. Thiobarbituric acid reactants were measured as an index of lipid peroxidation and malondialdehyde formation. Reversible binding of paraquat to lung homogenates was measured by equilibrium dialysis. Uptake of $^{14}C$-paraquat by lung slices was measured by a procedure similar to that described by Rose, Smith and Wyatt (Nature, 252: 314, 1974).

Major Findings: Treatment with a single dose of the beta adrenergic receptor stimulant, l-isoproterenol, markedly increased the toxicity of i.p. paraquat (Table 1), as evident from a 55% reduction in the LD50. Treatment with theophylline also increased paraquat toxicity. Multiple doses of the beta receptor blocking agent, dl-propranolol, protected rats moderately, as

evident from a 36% increase in the LD50. These findings suggest an involvement of the beta adrenergic receptor. A radomized experiment was therefore designed to compare the effectiveness of propranolol isomers in protecting rats from paraquat poisoning. There were 34 deaths from paraquat in 48 rats treated with saline. Only 17 rats died from paraquat among 48 rats treated with multiple doses of dl-propranolol. Treatment with l-propranolol was almost as effective (21 deaths in 48 rats) as treatment with d,l-propranolol; d-propranolol was less effective (28 deaths in 48 rats). The fact that d-propranolol was less effective than l-propranolol or D-propranolol supports the hypothesis that beta adrenergic blockade is at least part of the mechanism responsible for protection.

This laboratory has previously reported the finding that paraquat does not bind covalently to tissue proteins or other tissue macromolecules. We have found, however, that paraquat does bind reversibly to lung homogenates. The % binding to a 20% lung homogenate was about 62%. This in vitro binding to a lung homogenate was not affected by propranolol in vitro.

The uptake of paraquat by lung slices in vitro has been shown to be energy-dependent (Rose, Smith and Wyatt, Nature, 252: 314, 1974). Our experiments indicate that the uptake of paraquat by lung slices is inhibited appreciably by dl-propranolol in vitro. The inhibitor constant $K_i$ for dl-propranolol is approximately $1.4 \times 10^{-4}$M. l-Propranolol may be slightly more effective than d-propranolol as an inhibitor of paraquat uptake. Dichloroisoproterenol is about as effective as propranolol. Other beta adrenergic blocking agents were less effective than propranolol or dichloroisoproterenol in inhibiting the uptake of paraquat by rat lung slices.

Among other compounds tested for inhibition of paraquat uptake, imipramine and desipramine (DMI) were as effective as propranolol. Unlike propranolol, however, treatment with imipramine or desipramine did not reduce the 48-hr mortality from paraquat i.p.

Although propranolol in vitro inhibited paraquat uptake, there were no statistically significant differences in tissue paraquat concentrations 6, 24 and 48 hr after the i.p. injection of $^{14}C$-labeled paraquat between rats treated with multiple doses of propranolol or with saline. Decreasing concentrations of paraquat were found in the following tissues: lung > kidney >> liver > heart > muscle > brain > plasma.

Other investigators have suggested that paraquat-induced formation of the superoxide free radical, singlet oxygen, or peroxides is responsible for the pulmonary toxicity of paraquat. We have been unable to detect a paraquat-induced increase in diene conjugation of lung microsomal lipid, either from lungs of rats injected with paraquat i.p. or from lung slices incubated in vitro with high concentrations of paraquat, either in air or 95% $O_2$, 5% $CO_2$. Measurements of thiobarbituric acid (TBA) reactants, presumably malondialdehyde, in the incubation fluid did not reveal any significant increases resulting from incubation of lung slices with high concentrations of paraquat. Thus we have obtained no evidence in support of the view that toxicity is

caused by superoxide free radical formation and peroxide formation.

Significance to Biomedical Research and the Program of the Institute: The demonstration that l-isoproterenol and theophylline treatment increase the mortality from paraquat poisoning in rats may have clinical implications. It would seem wise for physicians to refrain from use of these bronchodilators in the treatment of paraquat poisoning. This caution is surprising since the use of a bronchodilator would seem to be a desirable symptomatic treatment for a person experiencing respiratory distress.

It is hoped that a better understanding of the mechanisms involved in paraquat poisoning will increase our insight into the mechanisms involved in the production of pulmonary edema from other causes.

Proposed Course of Project: TBA reactants will be measured in incubation fluid and in lung homogenates under a greater variety of conditions, in a continuing search for evidence of peroxide formation during the development of lung toxicity from paraquat. The studies of paraquat uptake by lung slices will be extended.

Proposed Course of Project: TBA reactants will be measured in incubation fluid and in lung homogenates under a greater variety of conditions, in a continuing search for evidence of peroxide formation during the development of lung toxicity from paraquat. The studies of paraquat uptake by lung slices will be extended.

Keyword Description:

diene conjugation of lung microsomal lipids
l-isoproterenol
paraquat
dl-propranolol
pulmonary edema, pulmonary toxicity
theophylline

Honors and Awards: None

Publications:

Maling, H.M., Webster, M.E., Williams, M.A., Saul, W. and Anderson,W.Jr.: Inflammation induced by histamine, serotonin, bradykinin and compound 48/80 in the rat: Antagonists and mechanisms of action. J. Pharmacol. Exper. Ther. 191: 300-310, 1974.

Table I

The effect of various treatments on the 48-hr LD50 values for paraquat, injected i.p. in male Sprague-Dawley rats, as calculated by probit analysis of pooled data.

| Treatment[a] | 48-hr LD50 for paraquat, injected i.p. | |
|---|---|---|
| s.c. | mg/kg (95% confidence interval) | μmole/kg |
| SALINE | 22.26 (21.61, 22.92) | 119.4 |
| dl-PROPRANOLOL | 30.27[b] (27.24, 33.64) | 162.5[b] |
| THEOPHYLLINE | 18.43[b] (15.06, 22.44) | 98.9[b] |
| l-ISOPROTERENOL | 10.04[b] (7.96, 12.67) | 53.9[b] |

[a]The saline-treated rats were 723 control rats in 54 experiments; these rats received 1-10 doses of saline s.c. in two days. Two to 10 doses of propranolol were injected in 230 rats treated with dl-propranolol. The first dose of propranolol each day was 25 mg/kg of the hydrochloride s.c.; the remaining doses were 18.75 or 20 mg/kg s.c., 1 1/2 to 2 1/2 hr apart. Only one dose of l-isoproterenol was given, 0.3 mg/kg (base) s.c. in 70 rats at the same time as the administration of paraquat i.p. ( 3 experiments). Only one dose of theophylline was given (50 mg/kg s.c. in 43 rats at the same time as the i.p. injection of paraquat - two experiments).

[b]These LD50 values are statistically significantly different from the corresponding value in saline-treated rats, $P < .05$.

Project No. Z01 HL 00852-03 LCP
1. Chemical Pharmacology
2. Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Ethanol, isopropanol, and $CCl_4$-induced liver toxicity.

Previous Serial Number: NHLI-47, NHLI 86

Principal Investigator: Dr. Harriet M. Maling

Other Investigators: Mr. Wilford Saul
Mrs. Martha A. Williams

Cooperating Unit: None

Project Descritpion:

Objectives: In our Annual Reports for 1971-72 and 1973-74, we reported that pretreatment of rats with four oral doses of ethanol over a period of two days potentiated the hepatotoxicity of a small dose of $CCl_4$, as evaluated by histologic grading and levels of plasma glutamic-pyruvic transaminase and liver triglycerides. Possible mechanisms for this potentiation have been examined. We have also considered the question whether the hepatotoxicity of other compounds is also potentiated by pretreatment with multiple doses of ethanol. These questions seemed important in view of the widespread social use of ethanol.

Methods Employed: Standard methods were employed.

Major Findings: The elevated plasma glutamic-pyruvic transaminase (GPT) activities induced by thioacetamide or dimethylnitrosamine, as well as those induced by $CCl_4$, were increased by pretreatment with four doses of ethanol. Plasma GPT activities after bromobenzene were not consistently enhanced by pretreatment with ethanol. The hepatotoxicity of allyl alcohol was not affected by pretreatment with ethanol. Plasma GPT activities were not elevated 24 hr after chlorpromazine (20-80 mg/kg ip) or isoniazid (100 and 150 mg/kg) in either untreated rats or rats pretreated with ethanol.

Diene conjugation of liver microsomal lipids from rats killed 30 min after the injection of a small dose of $CCl_4$ (0.1 ml/kg i.p.) was potentiated significantly by pretreatment with pyrazole and a single dose of ethanol, or by a single dose of isopropanol. Pretreatment with 4 doses of ethanol also tended to increase $CCl_4$-induced diene conjugation.

Significance to Biomedical Research and the Program of the Institute: In this study, we have analyzed several possible mechanisms responsible for a potentiated toxicity of $CCl_4$ and several other compounds in rats pretreated

with 4 doses of ethanol. Special interest arises from the finding that this potentiated hepatotoxicity is great when the blood alcohol levels are negligible. This suggests that physicians should consider the possibility of potentiated hepatotoxicity of $CCl_4$ and possibly other compounds in persons with a history of recent drinking, even though no alcohol can be detected in their blood.

Proposed Course of Project: This project has been terminated.

Keyword Description:

| | |
|---|---|
| blood alcohol levels | hepatotoxicity |
| $CCl_4$ | isopropanol |
| diene connugation | plasma glutamic-pyruvic transaminase (GPT) |
| ethanol | |

Honors and Awards: None

Publications:

Maling, H.M., Stripp, B., Sipes, I.G., Highman, B., Saul, W. and Williams, M.A.: Enhanced hepatotoxicity of carbon tetrachloride, thioacetamide, and dimethylnitrosamine by pretreatment of rats with ethanol and some comparisons with potentiation by isopropanol. Toxicol. Appl. Pharmacol., in press.

Maling, H.M., Eichelbaum, F.M., Saul, W., Sipes, I.G., Brown, E.A.B. and Gillette, J.R.: The nature of the protection against $CCl_4$-induced hepatotoxicity produced by pretreatment with dibenamine [N-(2-chloroethyl) dibenzylamine]. Biochem. Pharmacol. 23: 1479-1491, 1974.

Stripp, B., Sipes, I.G., Maling, H.M. and Gillette, J.R.: Dibenamine impairment of rat hepatic microsomal enzymes and its relation to hepatotoxicity induced by $CCl_4$ and dimethylnitrosamine. Drug Metabolism and Disposition 2: 464-468, 1974.

Project No. Z01 HL 00877-02 LCP
1. Chemical Pharmacology
2. Clinical Pharmacology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Hydrazines 1. Human isoniazid acetylation and metabolism.

Previous Serial Number: NHLI-43

Principal Investigators: Dr. J. R. Mitchell
Dr. U. P. Thorgeirsson
Dr. J. A. Timbrell

Other Investigators: Dr. W. R. Snodgrass
Dr. W. Z. Potter
Dr. M. Black
Dr. H. R. Keiser (NHLI:HE)

Cooperating Units: Dr. Thorgeirsson is supported by the American Lung Association.
Drs. Potter and Snodgrass are Research Associates in the Pharmacology-Toxicology Program, NIGMS
Dr. Black is a Fogarty International Fellow

Project Description:

Objectives: Isoniazid and iproniazid can produce severe and occasionally lethal hepatitis in man. Iproniazid was removed from clinical use because of this hepatotoxicity, and isoniazid hepatitis has become such a serious clinical problem that its use has been recently restricted.

In a prospective study (NHLI 43, Chest, in press) we examined isoniazid plasma concentrations and monthly liver function tests in 201 patients during one year of isoniazid preventive therapy for tuberculosis. About 20% of patients showed evidence of liver injury with abnormal SGOT or bilirubin values which subsided while the patients continued to take isoniazid. No correlation was found between patients that acetylated isoniazid slowly and those that had abnormal liver function tests. No anti-isoniazid antibodies were found and no correlation was seen between hepatic injury and antinuclear antibodies measured at the end of the study.

Subsequently, the U. S. Public Health Service conducted a surveillance study on 13,838 patients from 21 American cities to determine the incidence of isoniazid hepatitis. This study was abruptly terminated by then Assistant Secretary for Health, Dr. Merlin Duval, because of a high incidence of hepatitis (>2.3% in patients over age 50) and several fatalities. We were requested by the Tuberculosis Research Section, Center for Disease Control, U.S.P.H.S., to evaluate data as to etiology for 224 of the 13,838 recipients

of isoniazid who were suspected of having developed isoniazid liver injury (NHLI 43, Gastroenterology, in press). Some of the important findings from this retrospective analysis were: 1) isoniazid-related liver injury was indistinguishable biochemically (SGOT, bilirubin, alkaline phosphatase) and morphologically from iproniazid-induced liver damage or from other causes of acute hepatocellular injury such as viral hepatitis; 2) no clinical evidence for a hypersensitivity mechanism was apparent; 3) about 30% of the patients with hepatic reactions were residents of Honolulu and of Oriental ancestry--accordingly, as many as 90% of this group would be expected genetically to be fast acetylators of isoniazid whereas only 45% of black and white Americans are rapid acetylators.

To evaluate a possible correlation of susceptibility to hepatic injury and rapid acetylation of isoniazid, we have now genetically phenotyped 26 non-Oriental patients from the Public Health Service trial who had recovered from isoniazid hepatitis. To determine whether other differences occurred in the metabolism of isoniazid by rapid and slow acetylators of the drug, radiolabeled isoniazid and acetylisoniazid were given to normal volunteers and urinary metabolites were isolated and identified.

Methods Employed: The acetylation phenotypes of patients who had been diagnosed as having isoniazid hepatitis were determined using the standard sulfamethazine method. In metabolic studies patients were given $^{3}$H-isoniazid or acetyl-$^{3}$H-isoniazid and urine was collected for 48 hours and analyzed by quantitative radiochromatography. Urinary metabolites were identified by their $R_f$ values, by co-chromatography with synthesized authentic standards, by color reactions with spray reagents, by reverse isotope dilution analysis and by mass spectrometry.

Major Findings: Table I gives the clinical features and the acetylator phenotype for the 26 patients who had previously suffered "probable" or "possible" isoniazid liver injury. Of the 21 individuals in the "probable" category, 18 (86%) of them displayed the rapid phenotype for isoniazid acetylation whereas the expected frequency was 45%. Of the 5 subjects in the "possible" group, 3 (60%) were rapid acetylators.

Examination of the urinary metabolites of isoniazid and acetylisoniazid provided a possible explanation for the increased incidence of isoniazid hepatitis in patients who acetylate the drug rapidly. Fast acetylators hydrolyzed 44.4% of a dose of isoniazid to isonicotinic acid, whereas slow acetylators converted only 30.5% of a dose to isonicotinic acid (Table 2). This hydrolysis, ofcourse, simultaneously liberated stoichiometric amounts of the hydrazino moiety of isoniazid, and simple hydrazines are well-known hepatotoxins, mutagens and carcinogens. Thus, for any dose of isoniazid, rapid acetylators are exposed to 46% more of the free hydrazino moiety than are slow acetylators ($\frac{44.4\%-30.5\%}{30.5\%} \times 100$).

It is also apparent that the liberated hydrazino moiety is almost exclusively acetylhydrazine; i.e., almost all the excreted isonicotinic acid

came from acetylisoniazid rather than from isoniazid. Rapid and slow acetylators excreted equal amounts of acetylisoniazid and isonicotinic acid after administration of acetylisoniazid (Table 2). Accordingly, the differences in the excretion of acetylisoniazid and isonicotinic acid between fast and slow acetylators after administration of isoniazid must have resulted from differences in the rate of isoniazid acetylation; they could not have resulted from differences between the two groups in the disposition of these compounds. Similarly, rapid acetylators excreted as much acetylisoniazid and isonicotinic acid when receiving isoniazid as when receiving acetylisoniazid, demonstrating that they converted almost all the administered isoniazid initially to acetylisoniazid. In contrast, slow acetylators excreted much less acetylisoniazid and isonicotonic acid after receiving isoniazid, because more isoniazid ascaped acetylation and was eliminated free or as isoniazid hydrazones. Thus, the amount of isonicotinic acid formed in people receiving isoniazid, and therefore the amount of total hydrazino material liberated in the body, depends primarily on the formation of acetylisoniazid.

Recently we have demonstrated that acetylisoniazid and acetylhydrazine, but not isoniazid, are converted in rats in vivo to potent acylating agents that cause acute hepatocellular necrosis (see following reports). These data provide strong support for the hypothesis that isoniazid hepatitis in patients results from the liberation of acetylhydrazine in the body.

Significance to Biomedical Research and the Program of the Institute: The combination of clinical and animal studies provides a satisfactory explanation for the pathogenesis of isoniazid hepatitis. Given the seriousness of this adverse drug reaction and the crucial importance of isoniazid in treating tuberculosis, the significance of this work to the program of the institute is apparent.

Proposed Course of Project: We are attempting to prove this hypothesis in further human investigations (see following reports). If successful, we hope to develop either 1) alternative methods of isoniazid administration or 2) a new isoniazid analogue that could markedly reduce the hepatotoxicity of isoniazid therapy without altering pharmacologic efficicy in the treatment of tuberculosis.

Keyword Description:

| | |
|---|---|
| acetylation phenotypes | hepatitis |
| acetylhydrazine | iproniazid |
| acetylisoniazid | isoniazid |
| acylating agents | tuberculosis |

Honors and Awards: None

Publications:

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Table I
Acetylator phenotype and clinical features of 26 patients with probable or possible isoniazid liver injury.

| %Acetylated Sulfamethazine[a] | | Acetylator Phenotype[b] | Diagnosis[c] | Age Race Sex[d] | Peak SGOT or SGPT[e] | Peak Bilirubin[e] |
|---|---|---|---|---|---|---|
| Urine | Blood | | | | | |
| 93.6 | 66.1 | Rapid | Probable | 59 BF | 700 | 10.2 |
| 95.1 | 83.3 | Rapid | Probable | 53 WM | 1015 | 35.5 |
| 83.3 | 43.7 | Rapid | Probable | 48 BF | 1065 | 28.5 |
| 84.9 | 59.1 | Rapid | Probable | 69 BF | 1250 | 7.1 |
| 88.1 | 72.8 | Rapid | Probable | 56 WM | 1420 | 13.2 |
| 74.5 | 70.1 | Rapid | Probable | 60 WF | 1710 | 27.0 |
| 88.9 | 78.3 | Rapid | Probable | 53 BF | 2780 | 6.3 |
| 89.9 | 57.8 | Rapid | Probable | 49 BF | 2834 | 12.0 |
| 91.3 | 69.7 | Rapid | Probable | 59 WF | 975 | 21.2 |
| 87.1 | 61.3 | Rapid | Probable | 65 WM | 500 | 7.4 |
| 82.3 | 51.8 | Rapid | Probable | 26 BF | 700 | 7.5 |
| 91.4 | 58.0 | Rapid | Probable | 40 WM | 500 | 1.6 |
| 80.8 | 49.2 | Rapid | Probable | 35 WF | 160[f] | 0.6 |
| 77.2 | 57.6 | Rapid | Probable | 48 BF | 1118 | 7.0 |
| 90.4 | 48.1 | Rapid | Probable | 42 BF | 870 | 0.9 |
| 81.7 | 31.4 | Rapid | Probable | 37 BF | 650 | 1.3 |
| 88.7 | 49.3 | Rapid | Probable | 57 WF | 760 | 1.7 |
| 91.7 | 57.9 | Rapid | Probable | 54 WM | 490 | 1.1 |
| 40.0 | 5.8 | Slow | Probable | 49 WF | 660 | 25.3 |
| 65.2 | 6.2 | Slow | Probable | 61 WF | 638 | 9.5 |
| 32.7 | 4.3 | Slow | Probable | 64 WM | 260 | 0.7 |
| 82.1 | 29.4 | Rapid | Possible | 51 WM | 116 | 0.8 |
| 72.6 | 26.1 | Rapid | Possible | 47 BM | 87 | 0.4 |
| 85.9 | 60.4 | Rapid | Possible | 56 BM | 51 | 0.3 |
| 47.7 | 2.4 | Slow | Possible | 23 WF | 97 | 0.7 |
| 52.6 | 3.9 | Slow | Possible | 54 WF | 68 | 0.6 |

[a]Proportion of acetylated sulfamethazine 6 hr after drug administration to patients recovered from liver injury.
[b]Patients classified as rapid acetylators if the proportion of acetylated sulfamethazine was more than 70% in urine or more than 25% in blood.
[c]Probable or possible isoniazid liver injury.
[d]Age (yr), B (black) or W (white) race, M (male) or F (female) sex.
[e]Peak abnormalities during liver injury -- upper limits of normal, SGOT (45), SGPT (40), bilirubin (1.0).
[f]Diagnosis confirmed by liver biopsy.

Table II

% of Dose

| Drug | Patients Acetylation Rate (N) | INH | INH Hydrazones | AcINH | INA Derivatives | Estimated Acetyl Hydrazine | Estimated Hydrazine |
|---|---|---|---|---|---|---|---|
| AcINH | Fast (2) | --- | --- | 54.9±2.2 | 45.1±2.7 | 45.1±2.7 | --- |
| AcINH | Slow (3) | --- | --- | 53.8±1.2 | 46.2±1.1 | 46.2±1.1 | --- |
| INH | Fast (3) | 2.8±0.4 | 3.6±0.4 | 49.2±1.9 | 44.4±3.9 | 41.0±3.8 | 3.4±0.1 |
| INH | Slow (4) | 10.9±0.8 | 26.5±4.8 | 32.1±1.2 | 30.5±3.5 | 26.8±3.3 | 3.7±0.2 |

Project No. Z01 HL 00878-01 LCP
1. Chemical Pharmacology
2. Clinical Pharmacology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Hydrazines 2. Chemical synthesis of labeled compounds.

Previous Serial Number: None

Principal Investigators: Dr. S. D. Nelson
Dr. J. R. Mitchell

Other Investigators: Mr. George Corcoran
Mr. Christopher Conner

Cooperating Units: Dr. Nelson is a staff fellow in the Pharmacology Research Associate Program, NIGMS.
Mr. Conner is funded by the American Lung Association.

Project Description:

Objectives: Isoniazid and iproniazid can produce hepatitis in man. Elucidation of the mechanism leading to this toxic reaction required the synthesis of several specifically labeled hydrazine derivatives.

Methods Employed: Published synthetic procedures were employed whenever possible using commercially available reagents, stable isotopes, and radiolabeled compounds. All radioactive syntheses were preceded by "cold" synthesis of enough material for melting point and NMR analysis. Radiochemical purity of the prepared derivatives was determined by recrystallization to constant specific activity (sp. act.) followed by thin-layer chromatography (tlc). The plates were scraped in 0.5 cm bands and each fraction counted by liquid scintillation spectrometry in a Beckman LS-355 instrument. The more unstable hydrazines were purified immediately prior to use. The position of the tritium radiolabel in certain specifically labeled hydrazines was determined as described in the Results. Melting points were determined on a Thomas Hoover Uni-Melt instrument and nuclear magnetic resonance (NMR) spectra were determined on a Varian A-60.

Results:

1. Acetylisoniazid (AcINH)

a. $N^2$-Acetyl-isonicotinoyl-($^3$H)-hydrazide (AcINH-$^3$H). This compound was prepared in 92% yield by acetylation of generally tritiated isonicotinic acid hydrazide (INH-$^3$H), supplied by Amersham-Searle, with acetyl chloride in a mixture of ethyl acetate-glacial acetic acid and sodium bicarbonate.

Evaporation of the mixture yielded a residue which was extracted into chloroform, washed with a small amount of water and evaporated. The yellow crystal mass was recrystallized from methanol:ether (3x) to constant specific activity and finally from isopropanol:hexane (1x). Radiochemical (99.8) purity was further confirmed by tlc on silica gel using 2-propanol:methanol 70:30 as developing solvent, r.f. = 0.6, and scraping 0.5 cm bands as described under Methods.

b. $N^2$-Acetyl-isonicotinoyl-($^{14}C$)-hydrazide (AcINH-$^{14}C$) was prepared in the same manner using $^{14}C$-carbonyl-labeled INH (Amersham-Searle).

c. $N^2$-Acetyl-($^3H$)-isonicotinoyl hydrazide ($^3H$-AcINH) and $N^2$-acetyl-($^{14}C$)-isonicotinoylhydrazide ($^{14}C$-AcINH) were synthesized by the same procedure using $^3H$-methyl and $^{14}C$-carbonyl-labeled acetyl chloride, respectively.

2. Acetylhydrazine (AcHz)

a. Acetyl-($^{14}C$)-hydrazine ($^{14}C$-AcHz) was prepared as the fumarate salt by transacetylation under reflux of carbonyl-labeled ethyl acetate-$^{14}C$ with hydrazine hydrate dissolved in ethanol. After refluxing several hours fumaric acid was added and upon cooling $^{14}C$-AcHz fumarate was crystallized. Recrystallization to constant sp. act. from ethanol (x3) and then from methanol:ether (x1) gave the desired product, m.p. 122-123°. Radiochemical purity was confirmed by tlc on Avicel developed in 4:1:1 n-butanol:ethanol: 0.4N $NH_4OH$, r.f. = 0.49. The product was recrystallized each time immediately prior to use since the compound slowly decomposed even under a nitrogen atmosphere in a dessicator maintained at -15°.

b. Acetyl-($^3H$)-hydrazine ($^3H$-AcHz) was prepared as the hydrochloride salt by the following sequence of reactions. t-Butyl carbazate (Aldrich) was reacted with acetic-($^3H$)-anhydride (New England Nuclear) in methylene chloride to yield $N^2$-acetyl-($^3H$)-t-butyl carbazate which was subsequently hydrolyzed in dilute methanolic HCl at room temperature. Evaporation yielded a hygroscopic crystalline mass which was recrystallized to constant specific activity (4x) from methanol:ether mixtures to give white rhombic crystals, m.p. 131-133°C. Radiochemical purity > 99% was further confirmed by tlc on Avicel-F using n-butanol:ethanol:0.4N $NH_4OH$ (4:1:1) as developing solvent, r.f. 0.48, and scraping 0.5 cm bands. Like AcHz fumarate, this salt slowly decomposed and had to be recrystallized immediately prior to use.

3. $N^1$,$N^2$-acetylhydrazine (DiAcHz)

$N^1$,$N^2$-Acetyl-($^3H$)-hydrazine (DiAcHz-$^3H$). This compound was synthesized by the acetylation of hydrazine hydrate with a slight molar excess (2.2:1) of acetic-$^3H$-anhydride. The product was recrystallized to constant sp. act. (x3) from methanol:ether and radiochemical purity confirmed by tlc as described for AcHz.

4. $N^2$-Isopropyl-isonicotinoylhydrazide (IpINH)

a. $N^2$-Isopropyl-isonicotinoyl-($^3$H)-hydrazide (IpINH-$^3$H). This hydrazide was synthesized by the reductive alkylation of INH-$^3$H with acetone and hydrogen gas at 50 psi in a Paar hydrogenator using 10% Pd on charcoal as a catalyst. Recrystallization to constant sp. act. (x4) from methylene chloride-hexane gave the desired product, m.p. 111-113°C. Radiochemical purity was determined to be > 99.8% based on tlc on silica gel using chloroform:ethanol:acetic acid 15:2:0.1, r.f. = 0.59, and methanol-ethyl acetate 1:1, r.f. = 0.68.

b. $N^2$-Isopropyl-(2-$^{14}$C)-isonicotinoyl hydrazide (2-$^{14}$C-IpINH). This radiolabeled analog was prepared and analyzed using the same procedure described for IpINH-$^3$H using unlabeled INH and acetone (2-$^{14}$C).

c. $N^2$-Isopropyl-(1,3-$^{14}$C)-isonicotinoyl hydrazide (1,3-$^{14}$C-IpINH). This analog was synthesized by alkylation of the sodium salt of INH, generated in situ with an equimolar amount of sodium methoxide, with isopropyl iodide (1,3-$^{14}$C) (Amersham). Purification and radiochemical purity determinations were carried out as previously described.

d. $N^2$-Isopropyl-(2-$d_1$)-isonicotinoyl hydrazide (2-$d_1$-IpINH). This stable isotope analog was prepared by reacting $N^2$-isopropylidene-isonicotinoyl hydrazide (synthesized by condensation of INH and acetone) with deuterium gas generated at room temperature in situ from sodium borodeuteride and platinum oxide in deuterated methanol. The reaction took approximately 4 minutes. NMR of the resultant product showed complete loss of the multipletcentered at 3.25δ. Integration showed no incorporation of deuterium into any other portion of the molecule.

e. $N^2$-Isopropyl-(2-$^3$H)-isonicotinoyl hydrazide (2-$^3$H-IpINH). This material was prepared using the same procedure described for the deutero-analog using sodium borotritide. The compound was purified to constant sp. act. as previously outlined. Acid hydrolysis of the compound and chromatography of the resultant isonicotinic acid and isopropyl hydrazine showed that all of the label was present in the isopropyl side chain. Results from the deuterium experiment show that the tritium is incorporated solely into the C-2 carbon of the isopropyl group.

5. Isopropyl hydrazine (IpHz).

a. Isopropyl-(2-$^{14}$C)-hydrazine (2-$^{14}$C-IpHz). This compound was prepared as the hydrochloride salt in the following manner. t-Butyl carbazate (Aldrich) was reductively alkylated with acetone (2-$^{14}$C) in a hydrogenator (50 psi-H ) at room temperature using 10% Pd on charcoal as a catalyst. The intermediate $N^2$-isopropyl-(2-$^{14}$C)-t-butyl carbazate was purified by recrystallation from n-hexane, m.p. 49-51°. This was then hydrolyzed at room temerature in methanolic HCl to give white needles of $^{14}$C-IpHz hydrochloride which were recrystallized to constant sp.act. from methanol:ether (x5), m.p. 110-112°C. The material was recrystallized immediately prior to use since the product was found to slowly decompose.

b. Isopropyl-(2-$^{3}$H)-hydrazine (2-$^{3}$H-IpHz). This tritiated analog was prepared by hydrogenation with tritium gas of the non-radioactive isopropylidene intermediate at room temperature and pressure using tris-(triphenylphosphine) chlororhodium as catalyst. This soluble catalyst has been shown to reduce double bonds without the side reactions of tritium exchange. The intermediate product was then hydrolyzed and purified as described above (5a).

Significance to Biomedical Research and the Program of the Institute: These syntheses have made possible the elucidation of the mechanisms of isoniazid and iproniazid hepatitis (Z01 HL 00879-01 LCP, Z01 HL 00881-01 LCP).

Proposed Course of Project: Additional syntheses will be performed according to research needs.

Keyword Description:

hydrazines
synthesis

Honors and Awards: None

Publications: None

Project No. Z01 HL 00879-01 LCP
1. Chemical Pharmacology
2. Clinical Pharmacology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Hydrazines 3. Studies of reactive metabolites in vitro

Previous Serial Number: None

Principal Investigators: Dr. S. D. Nelson
Dr. W. R. Snodgrass
Dr. J. A. Timbrell
Dr. J. R. Mitchell

Other Investigators: Mr. Kenneth Greene
Mr. George Corcoran

Cooperating Units: Drs. Nelson and Snodgrass are staff fellows in the Pharmacology Research Associate Program, NIGMS

Project Description:

Objectives: Both acetylhydrazine and isopropylhydrazine are potent hepatotoxins in male rats. In vivo studies indicated that metabolic activation is necessary to produce the toxic effects (NHLI # 43 ). To further elucidate the mechanism for activating these hydrazines to toxic intermediates, we carried out a series of in vitro experiments with rat liver microsomes.

Methods Employed: Rats were pretreated with phenobarbital for four days (75 mg/kg, i.p.) before removal of livers and purification and isolation of microsomes. A second group was treated in the same manner except that cobaltous chloride was administered twice daily (30 mg/kg, s.c.) during the last two days of phenobarbital pretreatment. Incubations were carried out and the covalent binding of reactive intermediates was determined at various substrate concentrations and under varying conditions according to procedures previously described by this laboratory.

Gassing experiments were conducted in septum-sealed vessels using 100% $N_2$, 9:1 $N_2$:$O_2$, 9:1 CO:$O_2$ and air. Propane was trapped in septum-sealed vessels and determined by gas-chromatography on a Perkin-Elmer 900 gas chromatograph (Poropak Q, column temp. 150°; injector 80°; flame ionization detector 200°; $N_2$ carrier gas flow 50 ml/min; retention time of propane, 1.5 min). Acetic acid was determined on the same column at 170°C and had a retention time of 3.5 minutes. Ratios of tritium to carbon-14 in the bound and chromatographed metabolites was determined by the channels-ratio method. Gas chromatography-mass spectrometry (gc-ms) was performed using the same column conditions already described, and was coupled to an LKB-9000S mass spec-

trometer with an electron energy of 70 eV, accelerating voltage of 3.5 KV, and a trap current of 50 uA.

Specifically radiolabeled compounds were prepared as previously described, and the substrates used included $N^2$-acetyl-isonicotinoyl-($^3$H)-hydrazide (AcINH-$^3$H); $N^2$-acetyl-($^{14}$C)-isonicotinoyl hydrazide ($^{14}$C-AcINH); acetyl-($^{14}$C)-hydrazine ($^{14}$C-AcHz); acetyl-($^3$H)-hydrazine ($^3$H-AcHz); $N^2$-isopropyl-(2-$^3$H)-isonicotinoyl hydrazide (2-$^3$H-IpINH): isopropyl-(2-$^{14}$C)-hydrazine (2-$^{14}$C-IpHz); isopropyl-(2-$^3$H)-hydrazine (2-$^3$H-IpHz).

Major Findings: In the presence or absence of NADPH only small amounts of AcINH-$^3$H and $^{14}$C-AcINH were bound to liver microsomes in vitro (~ 0.11 nmoles/mg/15 min). In contrast, a substantial amount of covalent binding (0.55 nmoles/mg/15 min) occurred with acetyl hydrazine at 37° in the presence of microsomal enzymes, oxygen, and NADPH. The binding required NADPH and oxygen. It was almost abolished by heat inactivation of the enzymes and was inhibited by a carbon monoxide:oxygen atmosphere and SKF-525A. Glutathione, a naturally occurring sulfhydryl-containing tripeptide, was also found to substantially decrease the binding with concomitant increase in the formation of a glutathione conjugate, subsequently indentified as S-acetyl glutathione. Gas chromatographic analysis of the methanol extract after precipitation of the protein showed the presence of acetic acid in those incubations containing NADPH with much smaller (20%) amounts in incubations without NADPH.

Finally, an antibody against NADPH-cytochrome c reductase decreased the binding to nearly the same extent as it decreased the rate of cytochrome c reduction. These studies show, therefore, that the enzyme system activating acetylhydrazine is a cytochrome P-450 mixed function oxidase.

Kinetic analysis of the covalent binding of acetylhydrazine using various pretreatments showed that the binding of radiolabel was markedly increased by phenobarbital pretreatment, which potentiated necrosis and in vivo binding, whereas it was decreased by pretreatment with cobaltous chloride, which blocked both the necrosis and in vivo binding.

The $K_m$ for binding with all treatments was found to be approximately $10^{-3}$ molar while the $V_{max}$ for acetylhydrazine binding was 0.03 nmoles/mg/min for cobaltous chloride pretreated animals, 0.06 nmoles/mg/min for normals, and 0.11 nmoles/mg/min for phenobarbital pretreatment. When mixtures of methyl labeled $^3$H-AcHz and carbonyl labeled $^{14}$C-AcHz were used, the ratio of tritium to carbon-14 bound was 0.92 compared to the injected material, illustrating that the entire acetyl group was bound.

The same conditions outlined for the in vitro binding of acetylhydrazine were applied to isopropylhydrazine after first determining that 2-$^3$H-IpINH was not bound. The results parallel those of AcHz showing that IpHz is also activated by a P-450 system. The kinetic data for the binding reaction of IpHz are nearly identical to those found for AcHz except that the apparent $K_m$ for the binding is one-tenth that of AcHz ($10^{-4}$ molar compared to $10^{-3}$ molar). This may explain in part why IpHz is a more potent alkylating and

necrotizing agent in vivo than AcHz.

As observed for AcHz, the same extent of covalent binding of radiolabel to tissue macromolecules was found for both 2-$^{3}$H- and 2-$^{14}$C-labeled IpHz, $^{3}H/^{14}C = 0.94$. The ratio found for the propane evolved in the in vitro incubations showed $^{3}H/^{14}C = 0.96$. Even more importantly, phenobarbital pretreatment, which increased in vitro binding, increased the amount of propane evolved. Similarly, cobaltous chloride pretreatment, which decreased the binding reaction, decreased the amount of propane evolved.

Significance to Biomedical Research and the Program of the Institute: These results demonstrate that acetylhydrazine and isopropylhydrazine are converted by hepatic P-450 oxidases to potent acylating and alkylating agents. These chemically reactive metabolites probably cause the serious hepatitis that occurs in patients treated with isoniazid and iproniazid. The possibility that these metabolites may be carcinogenic should also be considered. The propane studies and the $^{3}H/^{14}C$ ratio studies are consistent with the hypothesis that the reactive metabolites are radical species.

Proposed Course of Project: Completed. Manuscripts are in preparation.

Keyword Description:

acetylhydrazine
in vitro covalent binding
isopropylhydrazine
reactive metabolite
specific radiolabel

Honors and Awards: None

Publications: None

Project No. Z01 HL 00880-01 LCP
1. Chemical Pharmacology
2. Clinical Pharmacology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Hydrazines 4. Studies of reactive metabolites in rats.

Previous Serial Number: None

Principal Investigators: Dr. J. A. Timbrell
Dr. S. D. Nelson
Dr. W. R. Snodgrass
Dr. J. R. Mitchell

Other Investigators: Mr. Kenneth Greene
Mr. George Corcoran

Cooperating Units: Drs. Nelson and Snodgrass are Research Associates in the Pharmacology-Toxicology Program, NIGMS.

Project Description:

Objectives: We have previously postulated that the hepatic necrosis caused by isoniazid and iproniazid results from the liberation and subsequent metabolic activation of their hydrazino moieties to acylating and alkylating intermediates in the body (Z01 HL 00877-02 LCP, Z01 HL 00878-01 LCP, Z01 HL 00879-01 LCP). We now compare the rates of metabolism of hydrazino compounds by the proposed toxic pathways in rats, and the extent of hepatic necrosis and covalent binding.

Methods Employed: The following specifically radiolabeled derivatives have been prepared: 1) Acetylisoniazid-$N^2$-acetyl-isonicotinoyl ($^3$H-ring-labeled) hydrazide, AcINH-$^3$H; $N^2$-acetyl-isonicotinoyl ($^{14}$C-carbonyl-labeled) hydrazide, AcINH-$^{14}$C; $N^2$-acetyl ($^3$H-methyl-labeled) isonicotinoyl hydrazide, $^3$H-AcINH; $N^2$-acetyl-($^{14}$C-carbonyl-labeled) isonictinoyl-hydrazide, $^{14}$C-AcINH. 2) Acetyl hydrazine-acetyl-($^3$H-methyl-labeled)-hydrazine-hydrochloride, $^3$H-AcHz; acetyl ($^{14}$C-carbonyl-labeled) hydrazine fumarate, $^{14}$C-AcHz. 3) Iproniazid-$N^2$-isopropyl-isonicotinoyl ($^3$H-ring labeled) hydrazide, IpINH-$^3$H; $N^2$-isopropyl-isonicotinoyl ($^{14}$C-carbonyl labeled) hydrazide, IpINH-$^{14}$C; $N^2$-isopropyl-(2-$^{14}$C)-isonicotinoyl hydrazide, 2-$^{14}$C-IpINH; $N^2$-isopropyl (1,3-$^{14}$C) isonicotinoyl hydrazide, 1,3-$^{14}$C-IpINH; $N^2$-isopropyl (2-$^3$H) isonicotinoyl hydrazide, 2-$^3$H-IpINH. 4) Isopropyl hydrazine-isopropyl (2-$^{14}$C) hydrazine hydrochloride, 2-$^{14}$C-IpHz; isopropyl (2-$^3$H) hydrazine hydrochloride, 2-$^3$H-IpHz (Z01 HL 00878-01 LCP). The derivatives were administered to rats intraperitoneally. Urine was collected and metabolites isolated by chromatography. Pulmonary expiration of acetone (2-$^{14}$C), $^{14}CO_2$ and $^{14}$C- or $^3$H-propane was trapped in a coupled series of three solutions: 2,4 dinitrophenylhydrazine ($^{14}$C-acetone trapped as hydrazone conjugate), ethanolamine:

2-methoxyethanol ($^{14}CO_2$), and diethyl ether at -78°C (propane). Urinary metabolites were identified by their $R_f$ values, by co-chromatography with synthesized authentic standards, by color reactions with spray reagents, by isotope dilution analysis and by mass spectrometry. Propane was detected in the ethyl ether trap by gas chromatography-mass spectrometry on a LKB 9000S using a Poropak Q column. Tritium to carbon-14 ratios ($^3H/^{14}C$) were determined by the channels ratio method in a Beckmann LS-355 scintillation spectrometer.

Major Findings: Table 1 compares the pulmonary expiration of $^{14}CO_2$ after administration of $^{14}C$-AcINH and $^{14}C$-AcHz to rats versus the extent of acylation of hepatic macromolecules. As expected, pretreatment of rats with phenobarbital, which potentiated hepatic necrosis, increased the amount of acylation and the expiration of $^{14}CO_2$. Conversely, cobalt chloride pretreatment, which reduced hepatic necrosis, decreased the extent of acylation and the expiration of $^{14}CO_2$. No expiration of $^{14}CO_2$ or covalent binding occurred after administration of AcINH-$^{14}C$. Thus, measurement of expired $^{14}CO_2$ after administration of $^{14}C$-acetyl-labeled acetylisoniazid ($^{14}C$-AcINH) or $^{14}C$-acetyl-labeled acetylhydrazine ($^{14}C$-AcHz) can be used as an index of the amount of acetylhydrazine that is converted to a chemically reactive, acylating species in vivo.

This conclusion is confirmed by analysis of the urinary metabolites from $^{14}C$-AcHz in the above experiments. Pretreatment of rats with an inhibitor of cytochrome P-450, cobalt chloride, markedly increased the total urinary excretion of radioactivity (Table 2) and decreased expiration of $^{14}CO_2$ (Table 1) because it apparently inhibited the P-450 oxidation of acetylhydrazine, as shown by the increases in the urinary excretion of free acetylhydrazine, diacetylhydrazine and the hydrazones of acetylhydrazine. The excretion of these metabolites are proportional to the availability (amount) of total acetylhydrazine in the body. Conversely, pretreatment with an inducer of cytochrome P-450, phenobarbital, decreased total urinary excretion of radioactivity and increased expiration of $^{14}CO_2$ because it induced the P-450 oxidation of acetylhydrazine, as reflected by the decreases in the urinary excretion of free acetylhydrazine, diacetylhydrazine and the hydrazones of acetylhydrazine. As expected, bis-p-nitrophenyl phosphate (BNPP) pretreatment, which prevents the hepatic injury caused by AcINH as well as its hydrolysis to acetylhydrazine, had no effect on the covalent binding after the administration of AcHz itself. However, the urinary excretion of AcHz metabolites after BNPP pretreatment is yet to be determined.

Table 3 compares the expiration of propane ($2-^3H$) after administration of isopropyl ($2-^3H$) hydrazine ($^3H$-IpHz) to rats versus the extent of alkylation of hepatic macromolecules. Pretreatment of rats with phenobarbital, which potentiated hepatic injury, increased the extent of alkylation, but unexpectedly decreased the expiration of propane-$2-^3H$. In contrast, cobalt chloride pretreatment decreased hepatic injury, alkylation and formation of propane ($2-^3H$).

In an attempt to explain this anomaly, the metabolism of IpINH-($^{14}$C-carbonyl) and 1,3-$^{14}$C-IpINH was examined (Tables 4, 5). In contrast to acetylisoniazid and acetylhydrazine, metabolism of isopropylisoniazid (iproniazid) and isopropylhydrazine by cytochrome P-450 oxidases presumably occurs at C-2 of the isopropyl group in addition to nitrogen oxidation, because acetylisoniazid (15.7%, Table 4), and acetone (1.9%, Table 5) were formed from isopropylisoniazid. Thus the effects of inducers and inhibitors of P-450 oxidases on propane expiration will be quite complex, and therefore correlations in vivo between covalent binding and propane expiration will be meaningless without a complete metabolite profile. This will necessitate a more comprehensive metabolic study of the fate of both IpINH and IpHz.

To help elucidate the mechanism leading to covalent binding, we have used the technique of administering mixtures of $^{3}$H and $^{14}$C isotopes of isopropylisoniazid and isopropylhydrazine. When a mixture of 2-$^{3}$H-IpINH and 1,3-$^{14}$C-IpINH was administered to rats, the covalently bound material was found to have a ratio of $^{3}H/^{14}C$ = 0.92 compared to the injected solution, and the expired propane gas had a similar ratio of $^{3}H/^{14}C$ (0.94). When a mixture of 2-$^{3}$H-IpINH and 2-$^{14}$C-IpINH was administered, the covalently bound material was determined to have a ratio of $^{3}H/^{14}C$ = 0.96 that of the injected material and the collected propane again had virtually the same ratio (1:1). These ratios show that neither the covalent binding nor the propane arise by way of C-2 oxidation. Furthermore, these results, coupled with the gas chromatographic and mass spectometric data on the expired propane, provide strong evidence that the covalently bound material retains the entire isopropyl group and probably arises by the same enzyme pathway that produces propane.

Significance to Biomedical Research and the Program of the Institute: These results provide direct evidence in vivo for the generation of chemically reactive, hepatotoxic metabolites from isoniazid (via acetylisoniazid) and iproniazid. They support the results obtained in vitro on the mechanism of hydrazine activation and covalent binding of reactive metabolites.

Proposed Course of Project: Further studies are to be carried out on the metabolism in vivo of IpINH and IpHz.

Keyword Description:

gas chromatography
hydrazines
mass spectrometry
metabolism in vivo
reactive metabolites

Honors and Awards: None

Publications: None

Table 1

Effect of treatment on in vivo hepatic covalent binding and $^{14}CO_2$ formation after AcINH-$^{14}$C, $^{14}$C-AcINH and $^{14}$C-AcHz

| Treatment | AcINH-$^{14}$C 200 mg/kg | $^{14}$C-AcINH 200 mg/kg | $^{14}$C-AcHz 20 mg/kg | AcINH-$^{14}$C 200 mg/kg | $^{14}$C-AcINH 200 mg/kg | $^{14}$C-AcHz 20 mg/kg |
|---|---|---|---|---|---|---|
| | covalent binding nmol/mg protein (6 hr after dose) | | | $^{14}CO_2$ - (% of dose expired in 6 hr) | | |
| None | < 0.05 | 0.20 ± .021 | 0.15 ± .021 | 0 | 9.8 ± 1.3 | 29.0 ± 1.7 |
| Phenobarbital | < 0.05 | 0.31 ± .021 | 0.19 ± .012 | 0 | 12.2 ± 1.5 | 35.3 ± 1.3 |
| Phenobarbital + Cobaltous chloride | - | 0.15 ± .039 | 0.09 ± .008 | - | 4.0 ± .70 | 22.0 ± 1.2 |

Table 2
Disposition of $^{14}C$-acetylhydrazine *in vivo* in rats

| | % dose excreted in 6 hr urine | | |
|---|---|---|---|
| | None | Phenobarbital (PB) | Cobalt chloride + PB |
| Total urinary metabolites | 21.7 | 17.2 | 39.6 |
| | % dose excreted in 6 hr urine | | |
| α-Ketoglutarate | 2.9 | 2.1 | 6.1 |
| Acetyl hydrazone pyruvate acetyl hydrazone | 4.9 | 4.0 | 8.7 |
| Acetyl hydrazine | 1.5 | 0.7 | 1.9 |
| Diacetyl hydrazine | 5.4 | 3.8 | 11.6 |
| Unknown metabolite | 1.2 | 0.8 | 0.8 |
| Total | 15.9 | 11.4 | 29.1 |

Table 3

Effect of phenobarbital and cobalt chloride on propane formation and alkylation of hepatic macromolecules 5 hours after administration of isopropyl-(2-$^{3}$H)-hydrazine hydrochloride (20 mg/kg as free base) to rats.

| Treatment | Covalent Binding nmol/mg protein | Propane-2-$^{3}$H % of dose |
|---|---|---|
| None | 0.35 ± .025 | 6.7 ± 0.4 |
| Phenobarbital (Pb) | 0.44 ± .032 | 3.6 ± 0.2 |
| Pb + Cobalt chloride | 0.24 ± .034 | 2.2 ± 0.1 |

Table 4

Metabolism and disposition of
$N^2$-isopropyl-isonicotinoyl-($^{14}$C-carbonyl)-hydrazine (IpINH-$^{14}$C)

| | % dose excreted (average of 3 rats) |
|---|---|
| 24 hr urine | 67.8 |
| 48 hr urine | 15.7 |
| carcass + feces | 4.7 |
| Total | 88.2 |

| | % of 24 hr urinary radioactivity (average of 3 rats) | % dose excreted |
|---|---|---|
| Isonicotinoyl glycine | 12.5 | 8.2 |
| Isonicotinic acid | 41.0 | 27.6 |
| Acetylisoniazid | 23.0 | 15.7 |
| Unidentified metabolite | 6.0 | 4.0 |
| Iproniazid | 11.9 | 8.5 |
| Total | 94.4 | 64.0 |

Table 5

Metabolism and disposition of $N^2$-isopropyl-(1,3-$^{14}$C)-isonicotinoyl hydrazide (1,3-$^{14}$C-IpINH) in the rat.

| | % of Dose Excreted (average of 3 rats) |
|---|---|
| Urine 24 hr | 23.9 ± 1.1 |
| Urine 48 hr | 11.7 ± 0.9 |
| Expired acetone 24 hr | 1.9 ± 0.3 |
| Expired acetone 48 hr | 0.0 - |
| Expired $CO_2$ 24 hr | 17.1 ± 1.2 |
| Expired $CO_2$ 48 hr | 7.0 ± 0.9 |
| Expired propane 24 hr | 11.5 ± 1.1 |
| Expired propane 48 hr | 1.4 ± 0.2 |
| Carcass and feces | 13.7 ± 0.8 |
| Total | 88.2 ± 7.1 |

Project No. Z01 HL 00881-01 LCP
1. Chemical Pharmacology
2. Clinical Pharmacology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Phenacetin-induced toxicity: N-oxidation of phenacetin

Previous Serial Number: None

Principal Investigators: Dr. Jack A. Hinson
Dr. Jerry R. Mitchell

Other Investigators: Mr. George Corcoran
Mr. Kenneth Greene

Cooperating Unit: Dr. Hinson holds a NHLI Postdoctoral Fellowship

Project Description:

Objectives: Previous studies have shown that a toxic metabolite of acetaminophen is formed during the metabolism of the drug in the liver by a cytochrome P-450-dependent mixed function oxidase. Evidence has been presented that this toxic reactive metabolite is N-acetyl-4-benzoimidoquinone which arises through N-hydroxylation of the parent drug followed by non-enzymatic rearrangement. Recently we found that phenacetin also causes centrilobular hepatic necrosis in hamsters. Since N-hydroxyphenacetin will spontaneously rearrange to N-acetyl-4-benzoimidoquinone, a known arylating agent, we examined the capacity of hepatic microsomes to N-hydroxylate phenacetin.

Methods Employed: N-hydroxyphenacetin was synthesized from p-nitro phenetole in two steps: a) reduction to the hydroxylamine derivative by zinc dust in the presence of ammonium chloride and b) acetylation of the hydroxylamine derivative by acetyl chloride in the presence of sodium bicarbonate. $^{14}C$-Acetyl chloride was used to synthesize the acetyl-$^{14}C$-derivative by a modification of the procedure. Microsomes were isolated from hamsters by standard procedures. All other methods were standard procedures.

Major Findings: N-Hydroxyphenacetin was synthesized and proof of structure obtained by electron impact mass spectroscopy.

Incubation of $^{3}H$-phenacetin with hamster liver microsomes led to the formation of N-hydroxyphenacetin. Proof of the structure was obtained by electron impact mass spectroscopy: the spectrum of the isolated material was identical to that of the synthetic standard. It showed a molecular ion having a molecular weight of 195 and major mass fragments corresponding to the losses of oxygen, $COCH_2$, and oxygen plus $COCH_2$.



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A quantitative assay for N-hydroxyphenacetin has been developed and its specificity established by recrystallization of the product to constant specific activity. The reaction rate of 0.5 mM phenacetin was about 0.2 nmoles per min per mg protein whereas the rate of de-ethylation of phenacetin was about 2.5 nmoles per min per mg protein. The reaction was dependent on NADPH and molecular oxygen and was inhibited by a carbon monoxide-oxygen atmosphere indicating that it was catalyzed by cytochrome P-450. Sodium fluoride significantly increased the rate of N-hydroxylation.

Pretreatment of the hamsters with 3-methylcholanthrene increased the rate of N-hydroxylation while pretreatment with either piperonyl butoxide or cobaltous chloride inhibited the reaction. Phenobarbital pretreatment did not affect the activity of the enzyme.

Significance to Biomedical Research and the Program of the Institute: The present studies demonstrate that phenacetin is N-hydroxylated *in vitro* and is a significant metabolite.

Proposed Course of Project: Since N-hydroxy-phenacetin is an important *in vitro* metabolite, the potential hepotoxicity and nephrotoxicity of this metabolite will be examined.

Keyword Descriptions:

cytochrome P-450
hepatotoxicity
N-hydroxyphenacetin
nephrotoxicity
phenacetin

Honors and Awards: None

Publications:

ANNUAL REPORT OF THE
LABORATORY OF CHEMISTRY
NATIONAL HEART AND LUNG INSTITUTE

July 1, 1974 through June 30, 1975

The work of the Laboratory of Chemistry might be somewhat artificially divided between applications (problem-solving) and "core" research. Most of the members of the Laboratory engage in both in varying proportions and again this year the emphasis has been on the former. Our major physical tools, gas and liquid chromatography, mass spectrometry, Fourier-transform nuclear magnetic resonance, and X-ray crystallography are usually applied in that order to structural analysis of compounds of biological origin brought to us from other groups in NHLI, NIH and occasionally from the outside. Success in such collaborations depends heavily upon our general knowledge, as organic chemists, of the properties and reactivity of organic compounds since several cycles of purification and analysis are invariably required before meaningful results begin to appear. We have found that the best results in such collaborations are achieved when both groups take the time to acquaint each other with the origin of the problem, details of isolation and, in our case, idiosyncrasies of the physical techniques we use. Special requirements of the problem then often point up the need for new approaches and dictate the directions of our "core" research. Our interest in chemical ionization-mass spectrometry was one case in point since many biological compounds refuse to give molecular ions under electron impact. Our laboratory, working on plans from the literature, arranged construction of the second such source ever built in 1971. Today such sources are in widespread use since they provide superior results, especially in such areas as drug and metabolite analysis. Field desorption, providing mass spectra of involatile materials, is another case--this year our shop has finally completed construction of a source and emitter conditioning apparatus.

These modern spectral techniques provide a vast amount of data on a compound, and it must be processed and compared to even more vast literature to identify these compounds with minimum time and effort. For this reason, we have been even more heavily involved with the Division for Computer Research and Technology this year via the Chemical Information System (CIS). This sytem provides, or will shortly provide: 1) access to the CBAC files of the ACS via structure, etc., 2) the X-ray crystallographic literature via the Cambridge Data file, 3) $^{13}C$ and $^{1}H$ nmr data, both to solve coupling constant/chemical shift problems and to access the literature, and 4) a 30,000 compound file of mass spectral data. Display and manipulation of structures is also provided. It is our hope to find ways to make this extensive service, which we have found so useful in our own work, available to the general public through DCRT facilities. A preliminary attempt to provide such a service with the mass spectra search portion of CIS, operating through the Aldermaston (England) Data Center, using a GE timeshare network has not lived up to its capabilities due to organizational difficulties but it has provided us with valuable experience in dealing with such large timeshare networks.

In the area of nuclear magnetic resonance we have programs underway involving relaxation time measurements on $^{13}C$ nuclei of normal abundance and in general we are acquiring experience running compounds in this beautifully straightforward mode. Similar studies on the $^{31}P$ nucleus as a nuclear probe are providing insights concerning the nature of the protein-lipid interaction in phospholipids.

The use of proton nmr has allowed us to demonstrate that the lymphatic node dye known as "alphazurine 2G" varies in composition according to its source. This may explain reactions experienced by some individuals during lymphography.

We have gained enough experience in liquid chromatography this year to begin to be able to define its utility in our group which, in the past, has been heavily involved in gas chromatography. It is clear that the two techniques are comparable and complementary in many ways. We have repeatedly collected fractions at the sub-microgram level, evaporated solvents and obtained satisfactory mass spectra on the products. This technique should become even more valuable when coupled to field desorption mass spectrometry and we will spend more effort on the latter technique next year. Clearly, an improved detector would be very desirable in liquid chromatography to remove the need for ultraviolet-absorbing groups. We intend to attempt to use an electrochemical detector developed last year for catecholamine assays for this purpose, but other ideas are also being considered. The catecholamine assays have been temporarily shelved until the return of the staff member who developed them (E. Whitnack).

Gas chromatography-mass spectrometry continues to be as important as ever. With it we have been able to identify a microscopic amount of triamcinolone in the human eye, identify metabolites of alkoxymethyl derivatives of barbiturates and dilantin, metabolites of retinoic acid, etc., and in addition, our instrument continues to do the major portion of the overdose analysis in the Washington metropolitan area (N. Law, Suburban Hospital).

In connection with last year's comments, the GT-40 interactive oscilloscope has this year been functioning well, as has the automated microfiche reader. In retrospect, however, there seems little need for computer operation of this latter device.

Our computer costs continue to be high, both due to storage of 30,000 mass spectra on disk and because of the extensive use of the IBM-360 by the X-ray facility. The former should be helped considerably by FDA's offer to support us at $5,000/month. Clearly, they find the system useful in their work. Very extensive support in these endeavors also comes from EPA via Dr. S. Heller. Regarding the X-ray usage of the IBM-360, minicomputers appear to be on the verge of taking over the bulk of this work and when the cost curves intersect, we will consider acquisition of the necessary hardware.

The Finnigan GC-MS-Computer system bought for us by DCRT has been transferred to Dr. B. Brewer (NHLI) who uses it exclusively for analysis of PTH derivatives by chemical ionization.

Work on insect pheromones has been more limited this year but synthesis of a new class of fire ant venoms has been completed and several such venoms have been tested for antigenic activity (H. Baer, Bur. Biol. Stand, FDA). They are inactive, however, suggesting that yet another component of the venom is the culprit in this sting which is becoming increasingly identified as an important public health problem in Southern regions of the United States. We shall attempt to separate and identify the true antigen this year.

In the past year, members of our Laboratory, often in collaboration with others, have:

1. Determined the sterol compositions of polyene resistant mutants of Aspergillus fennelliae and Cryptococcus neoformans (Kim and Kwon-Chung, NIAID).

2. Determined the structure of a pheromone of a caligo as z-β-farnesene using GC-MS (M. Blum, U. Georgia).

3. Used chemical ionization mass spectrometry to identify dipeptides released by the action of cathepsin C on large peptides (B. Halpern, Wollongong U., Australia).

4. Compared electron ionization, chemical ionization, field ionization, and field desorption in a series of biologically important molecules (J. Damico, FDA; H. Beckey, U. Bonn).

5. Further developed the abilities and data base of the Chemical Information Service in terms of mass spectra, etc. Initiated a worldwide effort to collect $^{13}C$ spectra from workers in this field and developed a search routine to access them. Over 2,000 have been collected to date (S. R. Heller, EPA; R. Feldman, DCRT).

6. Using $^{31}P$ nmr, showed that alteration of the charge on the protein portion of a lipoprotein does not disturb its basic vesicular structure (B. Brewer, NHLI).

7. Carried out $T_1$-temperature studies on the $^{31}P$ in HDL and recombined HDL. Differences were observed, casting doubt on the wisdom of using recombined particles in studies on HDL. Paramagnetic shifts of $Pr(NO_3)_3$ tend to prove that both are still mycellular.

8. Using $^{1}H$ nmr, proved that commercial preparations of the lymphatic node dye, alphazurine 2G, are variable in nature, perhaps explaining reactions to the dye (L. M. Kleinman and P. K. Hiranaka, Phar. Dev. Service, NIH).

9. Using $^{13}C$ nmr, elucidated the structure of a mycotoxin from Stachysbotrysa alba (E. Mazzola and R. Eppey, FDA).

10. Determined the structure of the glyoxal-acetonedicarboxylic acid condensation product as exo vs. endo using $^{13}C$ nmr (U. Weiss and K. Rice, NIAMDD).

11. Established the structure of the alkaloid casselsine by $^{13}C$ studies on its dehydrogenation product.

12. Developed direct methods-programs for X-ray analysis.

13. Determined the structure of a pyrolysis rearrangement product of a tetracyclic diketone (T. Lee, Walter Johnson High School, Heart Association Fellow).

14. Using very small crystals, solved the structure of a benzopyrene derivative.

15. Elucidated the structure of gardmultine, a dimeric indole alkaloid of m.wt. 1100, the largest molecule yet studied with direct methods (T. Akiyama, U. of Tokyo).

16. Written programs to approach the intractable problem of triclinic crystals with 2 molecules per assymmetric unit.

17. Begun to convert the complex IBM-360 X-ray programs to interactive form for use by non-specialists.

18. Developed a method for the identification and characterization of urushiol (poison ivy) standards using specific ion analysis by GC-MS (H. Baer and M. Gross, Bur. Biol. Stand. FDA).

19. Elucidated the structures of a series of metabolites of methoxymethyl and butyloxymethyl phenobarbital and dilantin using GC-MS (E. Baumel, EPA).

20. Helped to elucidate the structure of a glutathione conjugate of prostaglandin E. (L. Cagen and J. Pisano, NHLI).

21. Synthesized a series of new pyrollidine-ring containing fire ant venoms (M. Blum, U. Georgia).

22. Worked out the GC-MS analysis of all of the amino acids in the saccharopine cycle, in connection with a metabolic defect known as saccharopinuria (J. Dancis and J. Hutzler, NYU)

23. Worked on methods for identifying histidine containing peptides isolated from amino acid analyzers in connection with the structure of "elongation factor" (E. Maxwell and E. Tudor, NIAMDD).

24. Completed the structure and absolute configuration of both the alkaloid astrocasine and the drug viminol.

25. Identified a new pentacyclic triterpene, isomultiflavenol, in Benincasa hispida.

26. Identified new sulfur-containing iridoid glycosides in a series of carcinogenic plants.

27. Identified with GC-MS, 8 components of camponotus ants as a series of aliphatic secondary alcohols and phenylethanol and its esters with aliphatic acids (M. S. Blum, U. Georgia) and synthesized same.

28. Determined the conformation of products from the condensation of acetylacetone and benzylacetophenone (F. H. Greenberg, NYU, Buffalo).

29. Isolated with liquid chromatography and identified with MS iodo-derivatives of hydroxylpindolol and hydroxybenzylpropanolol. Enough radioactive material was collected by LC for further studies, (E. M. Brown and G. Aurbach, NIAMDD).

Project No. Z01 HL 01001-02 LC
1. Laboratory of Chemistry
2.
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Electrochemical Methods of Analysis and Synthesis

Previous Serial Number: NHLI-56

Principal Investigator: H. M. Fales

Other Investigators: R. Nicholson, National Science Foundation
E. Whitnack, Univ. of Cincinnati, Dept. of Medicine

Project Description:

Work has temporarily been discontinued on the electrochemical catecholamine assay due to Dr. Whitnack's departure. Upon her return, we will continue to develop and apply this technique. We had reached the point of being able to assay epinephrine and norephinephrine in plasma and to note the differences in their concentrations during stress.

A specially designed cyclic voltammetry sweep generator has been completed by our shop and with it we shall investigate the properties of a series of urushiol (poison ivy)-related compounds, as well as urushiol itself, to determine whether differences in antigenicity can be related to structure (H. Baer, Bur. Biol. Std.; FDA). The possibility that o-quinones react with sulfhydryl groups on proteins to form haptens has already led to the demonstration that the antigenic nature of urushiol on guinea pigs can be entirely eliminated by coapplication of mercaptoethanol.

In the near future, we intend to study such oxidations in non-aqueous systems as models of drug metabolism.

Keyword Descriptors: cyclic voltammetry, urushiol, poison ivy, drug metabolism

Honors and Awards: none

Publications: none

Project No. Z01 HL 01002-03 LC
1. Laboratory of Chemistry
2.
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Nuclear Magnetic Resonance of Natural Products

Previous Serial Number: NHLI-57

Principal Investigator: E. A. Sokoloski

Other Investigators: None

Cooperating Units: None

Project Description:

Nuclear Magnetic Resonance spectroscopy offers the researcher a powerful tool for structural investigations. Application of this technique to various classes of natural products has been the major endeavor of this investigator.

Applications and Results

A majority of time has been devoted to investigation of phospholipids by phosphorus-31 Fourier Transform NMR spectroscopy. By using the naturally-occurring phosphorus as a nuclear probe within the lipid system, we (collaborating with Dr. Brian Brewer-NHLI) have studied the spin lattice relaxation time as a function of temperature and pH in several model lipid systems and several lipoprotein systems.

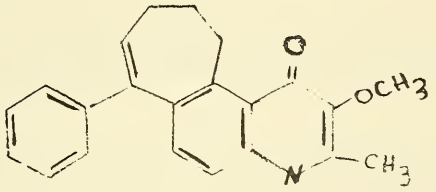
pH versus $T_1$ studies showed little or no alteration in relaxation time between pH 6.8-11.0, suggesting that alteration of the charge on the protein portion of the lipoprotein molecule does not disturb the basic vesicular structure of the lipoprotein. This could be interpreted as confirmation that the protein-lipid interaction is principally hydrophobic in nature.

$T_1$ versus temperature studies of natural high density lipoprotein (HDL) and recombined HDL showed differences in the nature of the relaxation time response to temperature changes. The native system gave increasing relaxation time with increasing temperature while the recombined system showed the opposite response in one case and a mixed response in a second case. No final conclusion has been drawn as yet, but, since $T_1$ is a measure of molecular motion which mirrors molecular organization, one is tempted to conclude that the particle is altered by the recombination process. This leaves one wondering if data obtained by many individuals on recombined particles can safely be extrapolated to native particles.

Titration of lipid models and native and recombined HDL also have been in progress. The praesodymium ion of $Pr(NO_3)_3$ is paramagnetic and causes a shift when complexed to a molecule. When added to sphingomyelin, a separation of the signal from inner and outer phosphorus of the bilayer is observed. Lyso lecithin, native HDL and recombined HDL do not show the separation, this being the expected behavior for a micelle. Qualitatively then, these seem to have the same molecular organization. However, we have noted a quantitative difference in the interaction of the last three species. Continuing experiments are needed to confirm this observation and delineate its cause.

A collaborative investigation with Dr. H. Fales, NHLI, and L. M. Kleinman and P. K. Hiranaka of the Pharmaceutical Development Service, NIH on the lymphatic node dye known as alphazurine 2G was concluded during the past year. Proton NMR of several different samples of the dye showed structural differences in the materials--all labeled as alphazurine 2G. Reports had been made that several patients injected with the dye for the purpose of obtaining lymphograms had experienced reactions to the material. The differences in structures observed could account for the reactions. A future publication will contain particulars of the investigation.

A structural study of material extracted from Melochia tomentosa plant used by natives of Curacao to relieve throat irratation and shown to be tumorogenic was completed. The study undertaken in collaboration with Drs. Fales and Silverton of NHLI and Dr. G. Kapadia of Howard University gave structure I for the extracted material.




Structure I

Keyword Descriptors: Melochia tomentosa, lipoproteins, phosphorus nmr, relaxation time, lymphatic node dyes

Publications:

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4. Assmann, G., Highet, R. J., Sokoloski, E. A. and Brewer, H. B. C-13 Nuclear magnetic resonance spectroscopy of native and recombined lipoproteins. Proc. Nat. Acad. Sci. (USA) 71: 3701-3705, 1974.

Project No. Z01 HL 01003-04 LC
1. Laboratory of Chemistry
2.
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Structure of Natural Products Using Instrumental Methods

Previous Serial Number: NHLI-58

Principal Investigator: H. M. Fales

Other Investigators: None

Cooperating Units: None

Project Description:

Two interesting pyrollidine variants of the major component of fire ant venom have been synthesized and tested, along with the ordinary piperidine analogs for antigenic activity (H. Baer, Bur. Biol. Std). Neither form has any activity and it is clear that another component of the venom must be responsible for its properties. More natural venom is being accumulated for isolation of the active component using guinea pig assays.

In collaboration with Drs. L. Cagen and J. Pisano, the structure of a glutathione adduct of prostaglandin $E_2$ has been elucidated. Key points in the structure proof were the appearance of a metastable ion in its mass spectrum showing loss of $H_2S$ (mass 34) and isotope ratios pointing to the presence of sulfur. Metastable defocussing should be especially helpful in such experiments and next year an effort will be made to automate their detection.

A series of dyes used in lymphangiography has been examined by $^1H$ nmr and several structures corrected or authenticated. The variability noted in the preparations of alphazurine 2G may be responsible for the side effects noted.

Poison ivy (urushiol) is responsible for a severe antigenic reaction in mammals, and in humans small differences in the side chain of the pentadecylcatechol unit cause very different responses. We have found that specific ion analysis of its trimethylsilyl ethers provides a sensitive and accurate method for assay of the various "standard urushiol" mixtures (H. Baer, FDA)

GC-MS has provided the structures of six new metabolites of a series of butoxymethylene and methoxymethylene barbiturates and dilantins. Interestingly, mass spectra of the two possible N-substituted dilantins were wholly dissimilar as were their G.C. retention times.

The Suburban Hospital quadrupole mass spectrometer has been modified by us in an attempt to improve its resolution and reliability. To date, its shortcomings in these regards have forced the Suburban group to continue to use our LKB spectrometer for emergency drug identification.

Keyword Descriptors: prostaglandins, overdoses, barbituates, fire ant, lymphagiography, alphazurine 2G, urushiol, trimethylsilyl ethers

Honors and Awards: Chromatographer of the Year - Washington Chromatography Group

Publications:

1. Tsai, S., Fales, H. M., and Vaughan, M. Inactivation of hormone-sensitive lipase from adipose tissue with adenosine triphosphate, magnesium and ascorbic acid. J. Biol. Chem. 248: 5278-5281, 1973.

2. Longevialle, P., Milne, G. W. A. and Fales, H. M. Chemical ionization mass spectrometry of complex molecules. XI. Stereochemical and conformational effects in the isobutane chemical ionization mass spectra of some steroidal amino alcohols. J. Amer. Chem. Soc. 95, 6666, 1973.

3. Heller, S. R., Pratt, A. W., Feldmann, R. J., Fales, H. M. and Milne, G. W. A. A conversational mass spectral search system IV. The evolution of a system ofr the retrieval of mass spectral information. J. Chem. Doc. 13: 130-133, 1973.

4. Brand, J. M., Fales, H. M., Sokoloski, E. A., MacConnell, J. G., Blum, M. S. and Duffield, R. M. Identification of mellein in the mandibular gland secretions of carpenter ants. Life Sci. 13: 201-211, 1973.

Project No. Z01 HL 01004-04 LC
1. Laboratory of Chemistry
2.
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Isolation and Characterization of Natural Products

Previous Serial Number: NHLI-59

Principal Investigator: H. A. Lloyd

Other Investigators: none

Cooperating Units:

Project Description:

1. Carcinogenic plant components - (with G.J. Kapadia, Howard University) Materials isolated from a number of plants (ex Acacia villosa, Krameri ixina, Paederia foetida, Diospyros virginiana) were examined - The structures of the new compounds were studied by mass spectrometry - Novel sulfur containing iridoid glycosides were found.

2. Pentacyclic triterpenes of Benincasa hispida. The main component was the alcohol, isomultiflorenol, not previously found in nature.

3. X-ray structure determination (with J. V. Silverton)

The structures and absolute configurations of the alkaloid astrocasine and of the drug Viminol were completed.

4. The NMR and mass spectra of Michael reaction products of acetylacetone and benzylacetophenone were studied to determine the conformation of the products (with F. H. Greenberg, NYU, Buffalo).

5. Insect pheromones (in collaboration with M. S. Blum, University of Georgia)

The compositions of glandular extracts of a number of insects were determined by combined gas chromatography-mass spectrometry. The suspected unknown components were synthesized for comparison.

a) Camponotus ants - Among 12 species studied, Camponotus clarithorax appeared especially atypical as to the variety of compounds (2,6-dimethyl-5-hepten-1-ol, 2-phenylethanol, citronellic, geranic, n-octanoic and n-nonanoic acids and their esters) not encountered previously in this genus.

b) Termites - new species were examined, mainly for their monoterpenes composition.

c) Millipedes - a new alkaloid was isolated from one specie.

d) Myrmecocystus ants - new terpene alcohols were characterized.

e) Myrmecia ants - Several species of these primitive Australian ants were studied (for C. P. Haskins, Carnegie Institution).

6. High Pressure Liquid Chromatography of Natural Products.

A considerable amount of time was devoted to the development of preparative HP liquid chromatography techniques for the separation of biological materials, drugs, natural products (terpenes, alkaloids, steroids, flavones). For example, metabolites of monobutoxymethyl dilantine and of monomethoxymethylphenyl barbiturate were collected and identified.

Radioactive iododerivatives of hydroxypindolol and hydroxybenzylproponolol were also separated and collected (for E. M. Brown NIAMDD). The technique is especially suitable for air or heat sensitive materials such as the Iridoid glycosides of Paederia foetida or the carcinogenic polyphenols of Acacia, Krameria and Diospyros.

Keyword Descriptors: high pressure liquid chromatography, insect pheromones, mono and triterpenes

Publications:

Brand, J. M., Blum, M. S., Lloyd, H. A. and Fletcher, D. J. C. Monoterpene hydrocarbons in the poison gland secretion of the ant Myrmicaria nataleusis. Ann Ent. Soc. Am. 67: 525-526 (1974).

Lloyd, H. A., Blum, M. S., and Duffield, R. M. Chemistry of the male mandibular gland secretion of the ant camponotus clarithorax. Ins. Biochem. in press.

Silverton, J. V., and Lloyd, H. A. The crystal and molecular structure of the non-morphinoid narcotic analgesic, Viminol. Acta Cryst. in press.

Project No. Z01 HL 01005-04 LC
1. Laboratory of Chemistry
2.
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: X-ray structural R&D for physiologically important molecules.

Previous Serial Number: NHLI-60

Principal Investigator: J. V. Silverton

Other Investigators: T. Lee, C. Kabuto, T. Akiyama

Cooperating Units: None

Project Description:

PURPOSE:

Investigations by X-ray crystallography of molecules of interest chemically or physiologically with emphasis on problems where other methods are inconclusive. Development of methods for extending and simplifying the techniques of direct methods.

SUMMARY:

Both centrosymmetric and acentric crystals have been studied by direct methods and further development and study of new methods have been carried out.

a) A pyrolysis rearrangement of tetracyclo $(4,3,0^{1.8},0^{2,10},0^{3,7})$tridecane-5-12-dione (with T. Lee).

Mr. Lee, who had just graduated from high school and was a recipient of a Heart Association fellowship, worked with the principal investigator on this project. The structure was solved by our own direct methods programs.

b) The structure of benzo-6,7,7a,8-tetrahydrobenzo[a] pyrene.

This structure was solved to resolve a disagreement as to stereochemistry which could not be settled by other means. Although the crystals were very much smaller than the optimum and the X-ray data are consequently not as accurate as usual, the structure was readily solved and is being refined.

c) The structure of gardmultine (with T. Akiyama)

This compound, an unsymmetrical dimeric alkaloid with a molecular weight of ca. 1100, represents the largest acentric structure we have studied and it is also one of the largest molecules ever attacked by direct methods. Currently we have not solved the structure but are carrying out necessary calculations.

d) The structure of chaetoglobosin, a cytotoxic metabolite of Chaetomium globosum (with T. Akiyama)

This is a substituted alternant 11-membered ring compound of only partially known structure and a molecular weight of 536. X-ray data has been collected and structure solution is about to start.

e) The structure of imerubrine (with C. Kabuto).

This structure represents a particularly intractable problem being a triclinic crystal with two parallel but independent molecules in the asymmetric unit. Since the basic assumption of direct methods, that the atomic positions may be regarded as numerically random, is far from true, the observed failure of all previously published direct methods approaches is not unexpected. We are now using the structure to develop and test a distinctly new approach in direct methods--quartet invariants (H. Hauptman, A.C.A. Meeting, Charlottesville, Va. 1975). We have written programs to implement the new method and currently we are working on the solution. Since quartet invariant methods may represent the greatest recent advance in direct methods, we are hopeful that development will radically simplify solution of structural problems.

f) Job control language writing programs (with T. Lee and G. W. A. Milne).

A start has been made on eliminating one of the worst barriers to the use of computer methods by non-specialists; the control language for IBM computers. Results are promising although currently incomplete.

Keyword Descriptors: X-ray, crystal, structure, organic

Publications:

Silverton, J. V., Milne, G. W. A., Eaton, E. E., Nyi, K., Temme, G. M. Structures of the [n.2.2] Propellanes I. 2-hydroxy[4.2.2] propellane p-nitrobenzoate. J. Am. Chem. Soc. 96: 7429-7432, 1974.

Silverton, J. V. and Lloyd, H. A. The structure of viminol. Acta Cryst. B31, in press 1975.

Akiyama, T. and Silverton, J. V. The structure of endo-tetracyclo [5.5.1.$0^{2,6}$.$0^{10,13}$] tridecane trione. Acta Cryst. B31 in press 1975.

Project No. Z01 HL 01006-05 LC
1. Laboratory of Chemistry
2.
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The characterization of natural matters

Previous Serial Number: NHLI-61

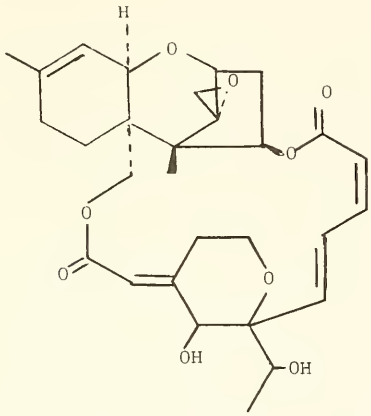
Principal Investigator: R. J. Highet, Ph.D.

Other Investigators: none

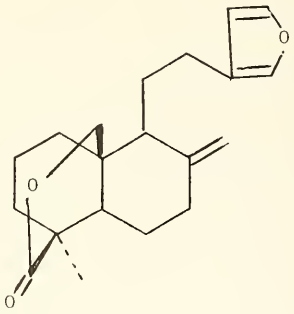
Cooperating Units:

Project Description:

In collaboration with Dr. Eugene Mazzola and Mr. Robert Eppley of the Food and Drug Administration, the structure of a mycotoxin of Stachysbotrys alba has been shown to be 1 by C-13 and proton nmr studies. The sesquiterpene and unsaturated acid moieties were readily established by comparison of the spectra with those of known materials, but the novel pyran systems could only be demonstrated by double resonance experiments.



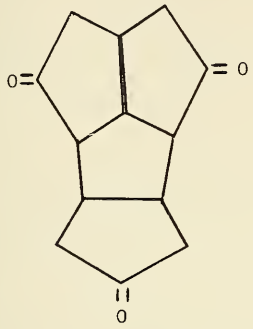

1




2

A detailed study of the C-13 characteristics of potamogetonin and related materials has established the structure as 2 and permitted the assignment of each observed resonance.

In a collaborative study with Dr. J. V. Silverton of this laboratory and Drs. U. Weiss and K. Rice of NIAMDD, the products of the condensation of acetone dicarboxylic acid and glyoxal have been studied. Among the structures examined, that of the exo ixomer of 3 was established by comparison of the C-13 spectrum with that of the known endo isomer.



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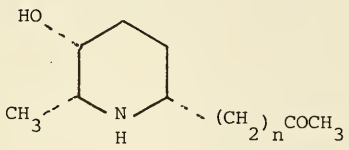

4

The structure of casselsine, 4, n = 12, has been established by comparison of the C-13 spectrum of its dehydrogenation product with that of the corresponding derivative of cassine (4, n= 10). Previously no facile method has been available to distinguish 4 from the structural isomer with the methyl and alkyl groups interchanged.

Keyword Descriptors: C-13 NMR; mycotoxin; diterpenes, natural products

Publications:

Assmann, G., Fredrickson, D. S., Sloan, H. R., Fales, H. M. and Highet, R. J. Accumulation of Oxygenated Steryl Esters in Wolman's Disease. J. Lipid Res. 16: 28-38, 1975.

Ziffer, H., Seeman, J. I., Highet, R. J. and Sokoloski, E. A. Carbon-13 Nuclear Magnetic Resonance Characteristics of 3-Methyl-cyclohexane-1,2-diols. J. Org. Chem. 39: 3698, 1974.

Highet, R. J. and Sokoloski, E. A. Structural Investigations of Natural Products by Newer Methods of NMR Spectroscopy. Fortschr. Chem. Organ. Naturstoffe, 32, 120-166, 1975.

Highet, R. J. and Edwards. J. M. Analysis of the Carbon-13 NMR Spectrum of Phenalenone. J. Mag. Res., in press

Project No. Z01 HL 01007-04 LC
1. Laboratory of Chemistry
2.
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Application of Mass Spectrometry to Problems in Biochemistry

Previous Serial Number: NHLI-62

Principal Investigator: G. W. A. Milne

Other Investigators: none

Cooperating Units: none

Project Description:

Chemical ionization mass spectrometry is now firmly established as an analytical technique with considerable utility in the area of analytical biochemistry and work in this laboratory on the development of the method has been largely supplanted by efforts to apply this technique to current problems. At the same time, we have undertaken the development of field desorption mass spectrometry, a newer technique which has some advantages over both electron ionization and chemical ionization mass spectrometry. Electron ionization mass spectrometry is still being used heavily in a multitude of problems.

In collaboration with Drs. Kim and Kwon-Chung of NIAID, the sterol compositions of polyene resistant mutants of Aspergillus fennelliae and Cryptococcus neoformans have been established. In each case, the various sterols were identified by combined gas chromatography-electron ionization mass spectrometry.

The same analytical technique has been used with a number of biologically active insect secretions, supplied by Dr. M. Blum of the University of Georgia. In this way, for example, a pheromone from the scent glands of butterflies of the species caligo has been identified as z-β-farnesene.

In collaboration with Dr. B. Halpern of Wollongong University, Australia, chemical ionization mass spectrometry has been used as a means of identifying the dipeptides released successively from large peptide chains by the action of cathepsin C.

Keyword Descriptors: computers, data bases, mass spectral data, CMR data, X-ray diffraction data, computer networks

Publications:

1. Heller, S. R., Koniver, D. A., Fales, H. M. and Milne, G. W. A. Conversational Mass Spectra Search System. Anal. Chem., 46: 947-950, 1974.

2. Heller, S. R., Feldmann, R. J., Fales, H. M. and Milne, G. W. A. A conversational mass spectral search system. IV. The evolution of a system for the retrieval of mass spectra information. J. Chem. Doc. 13: 130-133, 1973.

3. Heller, S. R., Fales, H. M., Milne, G. W. A., Feldmann, R. J., Daly, N. R., Maxwell, D. C. and McCormick, A. An experimental international conversational mass spectral search system. Adv. in Mass Spec. 6: 1037-1042, 1974.

Project No. Z01 HL 01008-04 LC
1. Laboratory of Chemistry
2.
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Use of Digital Computing in Problems in Biochemistry

Previous Serial Number: NHLI-63

Principal Investigator: G. W. A. Milne

Other Investigators: none

Cooperating Units: none

Project Description:

Work has continued in collaboration with EPA upon the Mass Spectra Search System (MSSS). In addition to this, a considerable effort has been invested in other aspects of the NIH Chemical Information System (CIS).

The MSSS is currently operating on the NIH PDP-10, which is used by workers in the Federal Establishment and also upon the G.E. International Computer Network, which is used by non-Federal and foreign research groups. Currently, there are some 140 users of the system. The data base now contains 28,000 mass spectra and should increase to include 50,000 in the next calendar year. All aspects of the system, even data collection, are operating relatively smoothly at present and it is hoped that this state of affairs will continue to improve so as to include the problems of expanding the data base at regular intervals.

A data base consisting of over 2,000 carbon-13 magnetic resonance spectra has been assembled and the programs necessary to search this data base are working. The number of spectra in this data base should soon reach about 3,000 which is well over 50% of all the spectra published and the value of this component of the Chemical Information System is already becoming clear. The X-ray crystallography sections of the CIS have been largely developed in DCRT and have functioned well for over a year.

An important link in the whole CIS is the sub-structure searching program. This will be used to facilitate the establishing of structure-literature and structure-experimental data relationships. This software was commenced at NIH but during the past year, the task of completing it was transferred to an outside contractor and should be finished during the coming year.

The mechanism of hydrogen rearrangement in esters upon electron, chemical and field ionization is under study with K. Levsen of the University of Bonn. Preliminary results suggest that rearrangement takes place in each case with a unique mechanism. The utility of various methods of ionization in the mass spectrometry of molecules of biological importance has been studied in collaboration with colleagues at the FDA and the University of Bonn. This study confirmed previous suspicions that the various methods tend to complement one another and that no single technique is universally superior to the others.

The use of carbon-13 as a label in biosynthetic studies is being explored. The label can be detected with precision by mass spectrometry and nuclear magnetic resonance spectroscopy and the possibilities of this approach are very promising.

Keyword Descriptors: computers, data bases, mass spectral data, CMR data
X-ray diffraction data, computer networks

Publications:

1. Kim, S. J., Kwon-Chung, K. J., Milne, G. W. A. and Prescott: Resistant Mutants of Aspergillus fennelliae: Identification of Sterols. Antimicrob. Ag and Chemother 6, 405-410, 1974.

2. Kim, S. J., Kwon-Chung, K. J., Milne, G. W. A., Hill, W. B. and Patterson: G. Relationship between polyene resistance and sterol compositions in Cryptococcus neoformans. Antimicrob. Ag and Chemother. 7, 99-106, 1975.

3. Schier, G. M., Halpern, B. and Milne. G. W. A. Characterization of Dipeptides by electron impact and chemical ionization mass spectrometry. Biomed Mass Spec 1, 212-218, 1974.

4. Fales, H. M., Milne, G. W. A., Winkler, H. U., Beckey, H. D., Damico, J. N. and Barron, R. Comparison of mass spectra of some biologically important compounds as obtained by various ionization techniques. Anal. Chem. 47, 207-219, 1975.

ANNUAL REPORT OF THE
LABORATORY OF KIDNEY AND ELECTROLYTE METABOLISM
NATIONAL HEART AND LUNG INSTITUTE
July 1, 1974 through June 30, 1975

The Laboratory of Kidney and Electrolyte Metabolism has made a number of significant advances in the elucidation of the mechanism and control of electrolyte transport in kidney, toad bladder, avian erythrocyte, and heart muscle. Only the first three will be summarized in the annual report. That concerning excitation-contraction coupling in heart muscle is discussed in detail in an appended individual summary.

Isolated segments of renal tubules

The technique of perfusing isolated segments of renal tubules in vitro, developed in this laboratory, has proved valuable as a means of studying kidney function. As detailed in previous reports, direct study of various previously inaccessible nephron segments has revealed a surprising diversity of function among the different parts of the tubule (of which there are eight in mammalia) and has uncovered a number of unexpected processes. Important examples include: 1) an active chloride transport system discovered in the thick ascending limb of Henle's loop, 2) the action of diuretics, which were found to inhibit chloride transport in this segment, 3) characterization of the interaction of vasopressin and the cyclic AMP system in the cortical collecting tubule, 4) analysis of the active sodium and potassium transport system in the cortical collecting tubule and of the organic acid and glucose transporting systems in proximal tubules, and 5) studies of the complex mechanism governing fluid absorption in the proximal tubule. The latter continues to be emphasized in our present work.

Transport in the proximal tubule accounts for over half the reabsorption of glomerular filtrate. Despite extensive study in vivo using micropuncture techniques there is little agreement on the mechanism of fluid transport. The isolated preparation provides for more stringent control of the experimental conditions than does micropuncture and for this reason is an especially useful approach to this difficult and important problem. In our initial studies of the isolated perfused rabbit proximal convoluted tubule, serum ultrafiltrate was used as perfusate with rabbit serum in the bath. Subsequently, artificial solutions were developed which supported fluid absorption as well as did serum and ultrafiltrate, and have the advantage that their composition can be more easily manipulated. Using these solutions, we found that certain organic solutes are important for fluid absorption. Glucose and alanine enhance fluid absorption when added to the perfusate and cause the voltage to increase, but they have no effect when added to the bath. In our present studies the mechanism of this effect was investigated. We find that α-methyl-D-glucoside

(a sugar) and cycloleucine (an amino acid) when added to the perfusate, also cause the rate of fluid absorption to increase. The latter compounds are known to be transported by kidney cells, but not metabolized. Therefore, it is transport, not metabolism of sugars and amino acids that is responsible for their enhancement of fluid absorption. When glucose and/or alanine are added to the perfusate, the tubule cells swell. Most likely, the non-electrolytes enter the cells during their transport which increases the intracellular osmotic pressure, and causes water to enter the cells. By analogy to the small intestine, which has been studied more extensively, the effect of glucose and alanine on fluid absorption probably is mediated by enhanced sodium transport. It is believed that the non-electrolytes are co-transported with sodium into the epithelial cells across the lumen surface. Entry of sodium into the cells is believed to be the first step in its transport and, though passive, to be rate limiting. Therefore, additional sodium entry into the cells, co-transported with the non-electrolytes, causes an increase in the rate of sodium transport which is in turn coupled to fluid transport. In previous studies, we found that there is a large passive back leak of glucose into the tubule lumen. A numerical analysis indicates that there is a significant glucose cycle composed of glucose which diffuses into the tubule lumen and is pumped out again. Since transport of non-electrolytes contributes to fluid absorption from proximal convoluted tubules, this process is important in understanding the function of the tubule.

Active transport of sodium is generally believed to be the driving force for reabsorption of fluid, as just discussed, but the evidence has been inconclusive and numerous alternative theories have been proposed. The importance of sodium transport was tested by removing sodium completely from the perfusate and bath (replacement with choline, tetramethyl ammonium, or lithium). Fluid absorption and voltage fell to zero, confirming the essential role of sodium. Removal of potassium from the bath has the same effect. Potassium is necessary for active sodium transport, as previously demonstrated in other tissues. Taken together, these results strengthen the conclusion that active sodium transport drives fluid absorption in proximal tubules. Complete removal of chloride from the perfusate and bath (replacement with nitrate or perchlorate) has virtually no effect, consistent with a passive role for chloride.

When bicarbonate is removed from the perfusate and bath, the rate of fluid absorption decreases by approximately one-third. Similar results were noted previously in micropuncture studies in rat kidneys. We tested the theory that as bicarbonate is reabsorbed from the tubule fluid and its concentration in the lumen falls, the bicarbonate concentration gradient itself drives fluid absorption. The effect is ascribed to an osmotic action of the relatively impermeant bicarbonate anions. We are unable to confirm this theory in isolated proximal tubules, however, since imposed

gradients of bicarbonate and methyl sulfate (another relatively impermeant anion) do not affect fluid absorption. Carbonic anhydrase is known to be important for bicarbonate reabsorption from proximal tubules. Therefore, we tested the effect of acetazolamide which is an inhibitor of carbonic anhydrase and is a mild diuretic. Acetazolamide ($10^{-5}$M) causes the rate of fluid absorption to decrease approximately as much as does removal of bicarbonate, suggesting that the effects are related. None of these experiments provides an explanation for the effect of bicarbonate, however, which remains to be determined.

Additional studies are underway to elucidate the mechanism of bicarbonate reabsorption and acidification in proximal tubules. Bicarbonate transport has been characterized in proximal straight tubules, using a new micromethod we developed for measuring total $CO_2$. We find that there is an active transport process which reabsorbs bicarbonate from the tubule lumen, despite an opposing back-leak of bicarbonate into the lumen. Straight proximal tubules from superficial and juxtamedullary nephrons were compared, and found to have essentially the same active transport rates. However, the tubules from the juxtamedullary nephrons are less permeable to bicarbonate than those from superficial tubules so that in the steady state the concentration of bicarbonate in the lumen is higher in the latter. The processes involved will be studied further in these and other nephron segments using this method as well as a microelectrode which measures pH.

Avian Erythrocytes

Studies performed in this laboratory with avian erythrocytes have added to our understanding of the mechanisms underlying the maintenance of normal cell volume in animal cells. It is generally accepted that animal cells behave like osmometers in that their volume is determined by the amount of osmotically active solute that they contain, especially the salts of sodium and potassium. Previously, it was believed that the intracellular sodium and potassium contents (and thus volume) were regulated by active transport via the classical ouabain-sensitive Na and K pump. Studies in this laboratory indicate that additional processes are important.

When the volume of duck erythrocytes is altered by changing the osmolality of the suspending medium, they spontaneously return to their original volume. In the case of swollen cells (i.e. those suspended in hypotonic solutions), shrinking back to the original size is accomplished by a large increase in potassium permeability allowing potassium salts to leak out of the cell, followed by water. Shrunken cells (i.e. those suspended in hypertonic media) swell back to their original volume, but the mechanism involved is not simply explained. Swelling back to original volume is due to net uptake of potassium salts, but not via the classical sodium and potassium pump. Ouabain does not prevent salt uptake and swelling. The swelling is also accompanied by a

large increase in potassium permeability, but this alone cannot explain the result, since it would cause potassium salts to leak out of, not into the cells.

The transport processes associated with cell swelling, with cell shrinking and the classical ouabain-sensitive cation pump have been characterized further. Cells incubated with 1 mM furosemide or cells in which intracellular chloride has been replaced with sulfate display normal sodium and potassium transport through the ouabain sensitive cation pump. These cells also shrink as do normal cells when swollen in hypotonic media. In contrast, furosemide treated cells and sulfate cells show no response to shrinkage in hypertonic media. The step at which the response is blocked remains to be determined. In other studies a technique was developed for preparing "ghosts" of the nucleated avian erythrocytes. These cells are normal in size, can maintain a 20-fold concentration gradient for potassium compared to the extracellular solution, and have a permeability to potassium similar to that of normal cells. Despite these properties, they fail to change permeability in response to hypotonicity or hypertonicity.

Investigation of the mechanism of recovery of shrunken cells has been facilitated by our previous observation that the apparently identical process can be induced by norepinephrine. It was found that compared to their normal volume in plasma, duck erythrocytes shrank when placed in isoosmotic salt solutions. Low concentrations of norepinephrine in the medium caused the shrunken cells to swell back to their normal volume. Dibutyryl cyclic AMP had the same effect, suggesting that the hormone operates through cyclic AMP. The role of cyclic AMP was confirmed by studies showing that norepinephrine elevates the concentration of cyclic AMP in duck red cells, as it elevates the permeability of the cells to potassium and the cells swell. In contrast, there is no change in cell cyclic AMP concentration associated with swelling in hypertonic solution. It seems likely that hypertonicity initiates its effect at a step after that at which cyclic AMP is generated.

Previously studies of ion transport in red cells have been limited for lack of techniques to measure directly the intracellular voltage, membrane resistance, and permeability to ions (especially anions, which exchange rapidly). We have now developed a technique which permits direct measurement of these parameters in a single amphibian red blood cell. The method involves immobilizing the cell within a narrow constriction of a glass pipet. The immobilized cell is readily penetrated with microelectrodes to measure voltage and electrical resistance. The cell is sealed well enough in the constriction so that ions pass through the cell rather than around it. Thus it is possible to measure permeability to isotopes and electrical resistance across the whole cell. In this experiment the isotopes or electric current pass

through portions of two membranes of the cell in series (one entering and one leaving the cell). The results with the two methods (puncture and transcellular measurements) are in good agreement. The major findings in Amphiuma red cells are: 1) the mean transmembrane voltage is -17 mV and varies directly with pH of the media, 2) the mean specific electrical resistance of the cell membrane is 100$\Omega cm^2$, 3) there is a high permeability to $^{36}Cl$, but this is in large part electrically silent and can be ascribed to exchange diffusion. This technique represents a major advance in methodology which will be widely used to examine the properties of red cells and probably many other types of cells, as well.

Toad Urinary Bladder

This section has continued to study the mechanisms by which hormones affect salt and water excretion. Previous work from this laboratory has established the thesis that vasopressin acts on responsive epithelial membranes by increasing the production and accumulation within the cell of cyclic AMP. Cyclic AMP, the "second messenger" of Sutherland and Rall, in turn elicits the effects of the hormone. The toad urinary bladder, which is analagous in many respects to the distal portion of the mammalian nephron, survives well in vitro. It has been used extensively in early work regarding the role of cyclic AMP, and in studies of factors that modify the response to vasopressin. For example, it has been shown that chlorpropamide, a sulfonylurea derivative that has considerable efficacy in the treatment of diabetes insipidus of pituitary origin, has an effect on the toad urinary bladder that is analagous to its clinical effect. Other studies have shown the interaction of adrenal steroid hormones, prostaglandins, adrenergic agents, and metabolic factors upon the response to vasopressin. Recent efforts have been directed toward elucidating the mechanism of action of cyclic AMP in the epithelial cells of toad bladder and kidney, and toward gaining an understanding of the function of the different types of epithelial cells in the epithelial membrane.

The effect of cyclic AMP in many tissues is thought to involve phosphoprotein metabolism. A protein kinase that is stimulated by cyclic AMP is widely distributed and has been shown to be present in toad bladder and mammalian kidney. Workers in another laboratory have reported that vasopressin (and cyclic AMP) stimulate a phosphoprotein phosphatase in toad bladder. We have been unable to confirm the report of stimulation of phosphoprotein phosphatase activity in the intact bladder, but have confirmed the presence of cyclic AMP stimulated protein kinase and phosphoprotein phosphatase activity in homogenates of toad bladder epithelial cells. The principle among many phosphoproteins affected by cyclic AMP has a molecular weight of 50,000 daltons in sodium dodecyl sulfate. Work has begun on the partial purification of the 50,000 dalton phosphoprotein. It appears to be a cytosolic protein. Our objective is to characterize the protein substrate

so that its function regarding the action of vasopressin will become evident. In addition, the purified substrate will enable us to study further the cyclic AMP stimulated protein kinase and phosphoprotein phosphatase and elucidate the role of these enzymes in response to vasopressin.

In other studies of the effect of cyclic AMP on phosphoprotein metabolism, we have used a suspension of separated renal cortical tubules, a technique developed in this laboratory several years ago. The bulk of cortical tubules are proximal tubules, one of the major sites of action of parathyroid hormone. It is established that parathyorid hormone acts by stimulating adenylate cyclase, and many of the renal effects of the hormone can be elicited by dibutyryl cyclic AMP. We have found that parathyroid hormone increases the incorporation of tracer phosphate into certain proteins of renal cortical tubules. The major effect on phosphorylation involves a protein with a molecular weight of about 50,000 daltons. Similar results occur in homogenates of renal cortex that are stimulated with cyclic AMP in the presence of $\gamma$-$^{32}$P-ATP. This is the first demonstration of an effect of parathyroid hormone on phosphoprotein metabolism. No evidence has been found that would indicate an effect of cyclic AMP on phosphoprotein phosphatase activity in renal cortex. Ultimately we hope to identify the major phosphoproteins affected by parathyroid hormone and define their role in the renal response to the hormone.

The epithelial cells of the toad bladder are morphologically heterogeneous. About 70 percent are "granular cells," 20 percent "mitochondria rich cells." If the cells are also functionally heterogeneous, as is likely, it is obviously important to know which cells are involved in the response to vasopressin. Two laboratories have interpreted electron micrographs of toad bladders as indicating that only the granular cells manifest increased water permeability in response to vasopressin. Another laboratory has removed epithelial cells from the toad bladder and has succeeded in separating the two major types of cells by density gradient centrifugation. They found that only the mitochondria rich cells respond to neurohypophysial hormones with an increase in cyclic AMP content. In view of the reports that only granular cells manifest increased water permeability in response to hormone, it has been suggested that cyclic AMP or another signal from the mitochondria rich cells stimulates water permeability in the granular cells. We have confirmed the separation of cell types by density gradient centrifugation. In our experience, the resulting cells are not suitable for study as intact cells. Therefore, we studied the activity in each type of cell of enzymes known to be involved in cyclic AMP metabolism in response to vasopressin. Granular cells are as rich in vasopressin sensitive adenylate cyclase activity and cyclic nucleotide phosphodiesterase activity as mitochondria rich cells. We have concluded that both cell types respond to vasopressin with increased cyclic AMP production.

Project No. Z01 HL 01201-01 KE
1. Kidney & Electrolyte
2. Membrane Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Study of the effect of cholera toxin on toad urinary bladder

Previous Serial Number: None

Principal Investigators: Joseph S. Handler, M. D.
Agens S. Preston

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: It is generally accepted that the toxin of Vibrio cholera (choleragen) acts on intestinal epithelial cells and on other tissues by stimulating adenylate cyclase activity. The resulting elevation of intracellular cyclic AMP levels is responsible for the typical response to the toxin, secretion of electrolyte and water in the small intestines, and in other tissues, responses resembling those elicited by specific hormones which stimulate adenyl cyclase. Recent reports indicate that choleragen binds to a specific glycolipid, the ganglioside $GM_1$ which is the binding site or receptor for choleragen in cell membranes. Once choleragen is bound to cell membranes, a temperature and time dependent reaction appears to be required for adenylate cyclase activation. It is the purpose of this study to examine the effect of choleragen on the toad urinary bladder. Normally, in vivo, vasopressin activates adenylate cyclase in the basal-lateral (blood surface) plasma membrane of the epithelial cells of the toad bladder. The resultant elevation of the intracellular concentration of cyclic AMP causes an increased rate of active sodium transport by the bladder and an increase in the permeability of the bladder to water. The objectives of the study are to gain information about the role of the epithelial cell plasma membrane in the activation of adenylate cyclase, and about the mechanism of action of the toxin.

Methods: After 18 hours of incubation with choleragen added to the solution bathing the mucosal (urinary) or to the solution bathing the serosal (blood) surface of the experimental bladder, the permeability to water and the rate of sodium transport by the

experimental and by the paired control tissue are measured using standard techniques. Vasopressin, cyclic AMP, or theophylline, the latter an inhibitor of the enzyme that destroys cyclic AMP, is added, and the sodium transport rate and water permeability response measured. $GM_1$ or other agents and conditions are imposed upon the choleragen treated tissue to assess their effect. Finally, adenylate cyclase is assayed in tissue that has responded to choleragen and in tissue in which the response to choleragen has been modified in a significant fashion.

Major Findings: Incubation with choleragen increases the response of the toad bladder to vasopressin and to theophylline, but does not increase the response to cyclic AMP. Therefore, it is likely that choleragen acts by increasing adenylate cyclase activity in the toad bladder, as in other tissues. Choleragen is more active when added to the solution bathing the mucosal surface than when added to the solution bathing the serosal surface. $5X10^{-11}$ M toxin is effective when added to the mucosal solution, but $5X10^{-9}$ M in the serosal solution is required for an effect. No effect of choleragen is detectable during the first 30 min. that it is present. By that time, however, it is bound to the tissue so that removing it from the bathing solution does not effect its subsequent action. $GM_1$ added to the same solution with toxin blocks its effect since it avidly binds the toxin in the solutions. In contrast, the addition of $GM_1$ to the serosal solution enhances the response of the bladder to choleragen added to the mucosal solution. A possible interpretation of this observation is that $GM_1$ added to the serosal solution is taken up by and incorporated into the basolateral plasma membrane. The $GM_1$ then migrates within the membrane to the mucosal surface where it serves as receptor to bind additional choleragen from the mucosal solution. If this interpretation is correct, the membranes must be fluid and there must be movement of molecules within the plasma membrane from the basolateral (serosal) and to the apical (mucosal) surface, despite the generally different properties of these membranes. The fact that choleragen added to the solution bathing the mucosal surface activates adenylate cyclase, an enzyme in the basolateral membrane, may also indicate movement of material in the membranes from one surface to the other.

Proposed course of project: The ability of $GM_1$ added to the solution bathing one surface, to enhance the response to choleragen added to the solution bathing the other surface will be examined further, using other combinations of agents, and applying additional controls. If it is confirmed that $GM_1$ and/or choleragen migrate within the plasma membrane, the factors required for and affecting the movement will be examined.

Keyword Descriptors: cholera toxin, ganglioside , $GM_1$

Honors and Awards: None

Publications:

Project No. ZO1 HL 01202-02 KE
1. Kidney & Electrolyte
2. Membrane Metabolism
3. Bethesda, Maryland

PHS-NHI
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Control of protein phosphorylation in toad urinary bladder

Previous Serial Number: NHLI-65

Principal Investigators: Gordon J. Strewler, M.D.
Dennis A. Ausiello, M.D.
Joseph S. Handler, M.D.

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: Previous studies in this laboratory have established that the actions of the hormone vasopressin on sodium transport and on water permeability in toad urinary bladder and in the renal collecting tubule of the rabbit are mediated by increases in the cellular levels of cyclic AMP. The effects of cyclic AMP in many tissues are thought to be the result of protein phosphorylation catalysed by cyclic AMP-dependent protein kinase. The state of phosphorylation of proteins is determined by the activity of protein kinases and phosphoprotein phosphatases.

In the initial phases of this study, we showed cyclic AMP dependent phosphorylation of several proteins in homogenates of toad bladder epithelial cells. The protein affected most by the cyclic nucleotide had a molecular weight of ∿50,000 daltons on SDS gels. A small effect of cyclic AMP on the rate of dephosphorylation of this protein was also evident, partially confirming results from another laboratory. We were unable to show any consistent effect of vasopressin on protein phosphorylation in intact cells.

The objectives of the study are:

1) To determine the distribution in subcellular fractions of toad bladder epithelial cells of cyclic AMP-dependent protein kinase activity, phosphoprotein phosphatase activity, and specific protein substrates for these enzymes.

2) To define the effect of cyclic AMP on protein dephosphorylation in this system.

3) To determine the role of changes in protein phosphorylation in the cyclic AMP-mediated effects of vasopressin in this tissue.

Methods: Phosphorylation of proteins in intact cells has been accomplished by incubating the cells with inorganic $^{33}P$. The bladders are then exposed to hormone or mediators (dibutyryl cyclic AMP, theophylline). Phosphoproteins are separated using polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS). Protein phosphorylation in homogenates and subcellular fractions occurs when the preparation is incubated with $^{32}P$-ATP in the presence of divalant cation.

Subcellular fractionation for these studies has employed differential dentrifugation and isopycnic sucrose density gradient centrifugation. Purity of fractions has been assessed by the assay of marker enzymes: 5' nucleotidase, adenylate cyclase, cytochrome oxidase, esterase, and glucose-6-phosphate dehydrogenase.

Phosphoproteins in the supernatant (cytosol) fraction have been separated using gel filtration, ammonium sulfate precipitation, and ion exchange chromotography.

Major Findings: A fractionation method has been developed which results in significant enrichment of plasma membrane, mitochondrial and cytosol marker enzymes in different fractions. No satisfactory marker for endoplasmic reticulum has been found. Autophosphorylation stimulated by cyclic AMP ($5X10^{-6}$ M) occurs mostly in the cytosol fraction. The principal among many soluble phosphoproteins affected by cyclic AMP is one of molecular weight 50,000 daltons (in SDS). There is some autophosphorylation of a 20,000 - 200,000Xg pellet probably composed of internal membranes, but there is little enhancement by cyclic AMP. No significant autophosphorylation of plasma membranes or mitochondrial fractions has been demonstrated. The rate of dephosphorylation in the cytosol fraction is slower than in the whole homogenate, but it appears to be enhanced significantly by cyclic AMP. The effects of cyclic AMP on dephosphorylation in other fractions has not been examined. The phosphoproteins in cytosol have been separated using the techniques mentioned above. The presence of protein phosphatase activity in the system has made it impossible to be certain that the label is not lost from a major protein species during separation.

It has not been possible to demonstrate a significant effect of vasopressin on protein phosphorylation in the whole tissue, although this has been reported by others.

Proposed Course:

1) Attempts are now underway to partially purify the soluble cyclic AMP dependent protein kinase from toad bladder epithelial cells. Using this enzyme, it will be possible to find potential protein substrates for phosphorylation in subcellular fractions that do not contain protein kinase activity.

2) Partial purification of the soluble phosphoprotein phosphatase is planned. This will help to explain the mode by which cyclic AMP stimulates dephosphoylation.

3) Studies of the 50,000 dalton substrate may elucidate its function. Current hypotheses are (a) that it may be the regulatory subunit of a protein kinase; if so it should display cyclic AMP binding activity, or (b) that it may be a component of the microtubular system, tubulin. It should then bind colchicine. Support for either of these hypotheses would be of importance in elucidating the path through which cyclic AMP acts as a "second messenger" for vasopressin.

Keyword Descriptors: Toad Bladder, Vasopressin, Cyclic AMP, Protein Kinase, Phosphoprotein Phosphatase

Honors and Awards: None

Publications: None

Project No. ZO1 HL 01203-01 KE
1. Kidney & Electrolyte
2. Membrane Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Separation by morphologic type and study of responsiveness to vasopressin of toad bladder epithelial cells

Previous Serial Number: None

Principal Investigator: Joseph S. Handler, M.D.
Agnes S. Preston

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: Previous work in this laboratory has established the thesis that vasopressin acts on responsive epithelial membranes by stimulating the enzyme adenylate cyclase, resulting in increased production and intracellular accumulation of cyclic-AMP. The toad urinary bladder used in the earlier studies, as well as other anuran and mammalian epithelial membranes that respond to vasopressin are morphologically heterogeneous (i.e. - contain more than one cell type). The possibility of performing more detailed and meaningful study of cyclic AMP metabolism in toad bladder cells was enhanced by a recent report from another laboratory that the two major types of epithelial cells (granular rich-70 percent of total cell population, and mitochondria rich - 20 percent of total population) could be separated from each other by density gradient centrifugation. The separation is established by examination of cells by electron microscopy and by three-fold enrichment of carbonic anhydrase activity in one band of cells on the gradient. Mitochondria rich cells have been found by other workers to be rich in carbonic anhydrase activity. It is the purpose of this study to confirm the density gradient separation of the cells and to examine the two cell types for vasopressin responsive adenylate cyclase and cyclic nucleotide phosphodiesterase, the enzyme that inactivates cyclic AMP by converting it to 5'-AMP.

Methods: The cells are separated by the method of Scott, Sapirstein and Yoder (Science, 184:797, 1974). The epithelial cells are removed from the bladder by incubating the tissue in calcium free Ringer solution containing 2 mM EDTA. The mixed

cell population is layered on top of a discontinuous gradient of Ficoll in EDTA Ringer. Cells are spun at 27,000 RPM in a Spinco SW-27 for 45 min. at 4°C and the material in the second and the third bands collected for further study after dilution in EDTA Ringer solution and centrifugation to remove the Ficoll.

The cells in every experiment are examined by phase contrast microscopy and in some experiments by electron microscopy. Carbonic anhydrase activity is assayed in the supernatant solution of sonicated cells using an aminco-Morrow stop-flow apparatus. The remainder of the cell material is used to study the responsiveness to vasopressin of the intact cells of each band or to study, in broken cell preprations, the activity of enzymes involved in cyclic AMP metabolism. Aliquots of intact cells are incubated in regular amphibian Ringer solution with or without arginine vasopressin. After 5 and 10 minutes an aliquot of control and hormone treated cells is added to TCA containing tracer $H^3$ - cyclic AMP (for extimation of recovery - 70-80%) and the cyclic AMP in the extracts separated by chromotography and assayed as described previously. For enzyme assays, aliquots of cells from each band are homogenized in a tris-magnesium buffer. Basal and vasopressin sensitive adenylate cyclase (whole homogenate or 1000Xg pellet) and cyclic nucleotide phosphodiesterase activity (1000Xg supernatant solution) are studied.

Major Findings: Electron micrographs reveal that band 2 is enriched in mitochondria rich cells and band 3 in granular cells, as reported. A large portion (25-50 percent) of the cells, however, are vacuolated or otherwise obviously damaged. The intact cells collected from the gradient have a variable and small increment in cyclic AMP content in response to vasopressin. These observations led to the conclusion that many of the cells collected from the gradient are not viable and are a poor preparation for study of function as intact cells. This is not surprising since the cells have been in calcium-free Ringer solution for three hours by the end of the Ficoll gradient separation. The material in band two uniformly has two to three times as much carbonic anhydrase activity (per mg. protein) as that from band three, confirming the enrichment of each band seen in electron micrographs.

Basal adenylate cyclase activity is the same in both bands. Vasopressin sensitive adenylate cyclase is slightly enriched in cells of band 3 (granular cells) which contain 20 percent more activity per mg. protein than do the cells in band 2. There is a similar enrichment in band 3 of cyclic nucleotide phosphodiesterase activity assayed at a low ($10^{-8}$ M) concentration of cyclic AMP, but no difference in activity between the two bands assayed at a high concentration of cyclic AMP ($10^{-4}$ M).

The results are interpreted as confirming that the two major cell types can be separated on a Ficoll gradient, but that the cells are too damaged for meaningful study. In contrast to the previous study (Science, 185:797, 1974) in which it was concluded that only the mitochondria rich cells respond to vasopressin with increased cyclic AMP levels, the enzyme assays of this study are interpreted as indicating that the granular cells respond to vasopressin with elevation of cyclic AMP production as well as the mitochondria rich cells.

Proposed Course of Project: Project is completed. Manuscript is in preparation.

Keyword Descriptors: Cyclic AMP, Granular Cells, Mitochondria Rich Cells, Adenylate Cyclase, Cyclic Nucleotide Phosphodiesterase

Honors and Awards: None

Publications: None

Project No. Z01 HL 01204-01 KE
1. Kidney & Electrolyte
2. Membrane Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Effect of parathyroid hormone on protein phosphorylation in rabbit renal cortical tubules

Previous Serial Number: None

Principal Investigator: Dennis A. Ausiello, M.D.
Joseph S. Handler, M.D.
Jack Orloff, M.D.

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: Work in several laboratories has shown that cyclic AMP exerts part or all of its effects within cells by stimulating the transfer of phosphate from ATP to certain proteins. This reaction is catalysed by a cyclic AMP dependent protein kinase. For several years this laboratory has been interested in the mechanism by which cyclic AMP alters transport and permeability and we are studying the effect of cyclic AMP on protein phosphorylation in toad urinary bladder. This project is a parallel study of the effect of parathyroid hormone on (PTH) and cyclic AMP rabbit kidney cortex. In this study, gel electrophoresis is used to separate phosphorylated proteins in an attempt to characterize the endogenous substrates for cyclic AMP dependent protein kinase(s) and to elucidate their role in the action of parathyroid hormone.

Methods: Rabbit kidney cortex homogenates were incubated at 23°C with $^{32}$P-γ-ATP under various conditions. The reaction was stopped by adding samples to boiling SDS (final concentration 1.0%) in 10 mM phosphate buffer pH 7.2. Samples were electrophoresed on polyacrylamide cylindrical gels, which were prepared as 5%-15% gradients for improved resolution. The gels were cut into 1 mm slices and the radioactivity in each slice determined by liquid scintillation counting.

In a second series of experiments, separated renal cortical tubules were prepared by the collagenase method previously described by this laboratory. The tubules were incubated at 23°C for various time periods with tracer inorganic $^{33}PO_4$ in standard

Krebs-Ringer solution without phosphate and gassed with 95% $O_2$-5% $CO_2$. Experimental manipulations were performed on aliquots and the reactions ended by the addition of boiling SDS. Samples were electrophoresed and processed as described above.

Major Findings: Studies with homogenates of cortex revealed several proteins in the 40,000-150,000 MW range whose phosphorylation was stimulated by cyclic AMP. The major effect was a peptide of ∿ 50,000 daltons. In order to interpret this result, it was important to determine whether similar changes occurred in intact cells stimulated by P.T.H.

The major phosphorylated peaks observed in the intact renal tubule cells were at ∿65,000 and ∿50,000 daltons with several smaller peaks between 100,000 and 150,000 daltons. A steady state level of phosphorylation was generally achieved after 45 min. incubation with $^{33}PO_4$. Phosphorylation stable through 105 min. Purified bovine PTH at a concentration of 100 U/ml stimulated the phosphorylation of the 50,000 dalton peptide (23% increase, $p <$ ,01) after 15 minutes of incubation. The phosphorylation of several proteins in the higher molecular weight range was also significantly stimulated. Preliminary data indicate that dibutyry cyclic AMP mimics this effect of PTH.

Proposed Course of Project: Emphasis will be placed on further characterizing the phosphorylated proteins affected by PTH in the intact cell. Attempts will be made to see whether variables believed to affect PTH action (eg. changes in $Ca^{++}$ and $Mg^{++}$ concentration alter the PTH-stimulated phosphorylation of proteins. In addition homogenates and subcellular fractions will be studied in order to localize and identify the phosphorylated substrates and to correlate them with the known actions of PTH.

Keyword Descriptors: Cyclic AMP, Protein Kinase, Parathyroid Hormone

Honors and Awards: None

Publications: None

Project No. ZO1 HL 01205-01 KE
1. Kidney & Electrolyte
2. Electrolyte Transport
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Regulation of Cation Permeability in Duck Erythrocytes

Previous Serial Number: NHLI-135

Principal Investigator: Dianne E. Robbie, Ph.D.

Other Investigators: None

Cooperating Units: None

Project Description:

In the erythrocytes of several avian species cation permeability is regulated by catecholamines and the electrolyte composition of the ambient medium. Previous work from this laboratory has demonstrated regulation of cell volume in duck erythrocytes. This is accomplished through the control of cation permeability by a volume-sensitive mechanism. Normal erythrocyte volume depends in vivo upon plasma levels of catecholamines, as indicated by the shrinkage to a new steady-state volume of erythrocytes incubated in catecholamine-free media or in plasma containing the ß-adrenergic blocking agent, propranolol. Addition of norepinephrine to shrunken "lower steady-state" cells results in an immediate increase in permeability to Na and K. In appropriate media the change in permeability results in net accumulation of cations, Cl, and osmotically obligated water. Except under special conditions, these volume changes are almost entirely associated with net uptake of KCl. Upon restoration of "normal" volume, cation permeabilities return to resting levels, presumably reflecting the intervention of a "volume sensor" which reverses the molecular changes initiated by norepinephrine.

A strikingly similar system is activated upon osmotic shrinkage of duck erythrocytes in hypertonic media. The responses to the two stimuli, catecholamines and hypertonicity, exhibit similar sensitivity to K and Na ion concentration in the medium and to drugs. Also, the maximal effects of the two stimuli on cation permeability are identical, within experimental error.

Previous evidence (1972-73) indicated that the permeability changes initiated by norepinephrine were a consequence of elevation of cellular cyclic AMP levels caused by activation of the membrane-associated adenylate cyclase of these cells. In contrast, permeability changes initiated by hypertonicity were not

accompanied by detectable changes in cyclic AMP content. Therefore, the two stimuli elicit the same ultimate effect, but a different chain of events.

Objectives: I. To determine whether the effects of norepinephrine and hypertonicity involve separate or common pathways for cation permeation;

II. To examine the possible interdependence of the mechanisms activated by the two stimuli;

III. To further identify the biochemical events initiated by hypertonicity which result in increased cation permeability.

Methods: Cation permeability was measured in suspensions of duck erythrocytes as $^{42}K$ influx or efflux, according to methods which have been previously described (Kregenow, F.M., J. Gen. Physiol. 58:372, 1971). A microcentrifugation assay has also been developed which provides greater speed and capability in tracer flux determination.

Major Findings: In the present studies, we have obtained further evidence that effects of norepinephrine and hypertonicity, though involving different initial steps, are mediated via a common final pathway. We find that a maximally effective degree of hypertonicity superimposed upon a maximally effective concentration of norepinephrine does not cause an appreciably greater effect on cation permeability than either stimulus alone. Furthermore, we have observed that the permeability response to hypertonicity is biphasic, decreasing when medium tonicity is increased beyond the level that results in maximal stimulation. Under these conditions the response to norepinephrine is inhibited in parallel with the response to hypertonicity. Norepinephrine-dependent cyclic AMP accumulation was also found to be progressively inhibited under these conditions, complicating interpretation of the result.

We have investigated the possibility that cyclic AMP levels may influence the response to hypertonicity in the erythrocyte. As a first step, we tested the effect of theophylline, which increases cyclic AMP concentration in many tissues by inhibiting its hydrolysis. Theophylline significantly inhibited the enhancement of K influx and K efflux that was caused by submaximal levels of hypertonicity, but had no effect on the enhancement of net K and water uptake. In contrast, theophylline potentiated the effect of submaximal concentrations of norepinephrine on K influx, and on the net uptake of K, Na and water. The theophylline effect was apparent only during the first few minutes of the

response to either stimulus. Therefore, if the cyclic AMP level is a determinant of the response it influences only the initial events leading to permeability changes.

Proposed Course of Project: 1) In order to test further whether the level of cyclic AMP modulates the response to hypertonicity, K fluxes will be measured during hypertonic stimulation in the presence of exogenous cyclic AMP and of other agents believed to alter cellular cyclic AMP levels. Cyclic AMP content of the cells will be measured. 2) Other areas of investigation under consideration are the possible involvement of cyclic GMP, of phosphodiesterases of differing specificity and of cyclic-nucleotide-activated phosphorylation/dephosphorylation mechanisms in volume regulation.

Keyword Descriptors: Erythrocytes, Cation Permeability, Catecholamines, Cyclic AMP, Hypertonicity, Volume Regulation

Honors and Awards: None

Publications: Kregenow, F.M. Robbie, D.E., and Orloff, J.: Effect of norepinephrine and hypertonicity on K influx and cyclic AMP levels in duck erythrocytes. (Submitted 3/75, Am. J. Physiol.)

Project No. Z01 HL 01206-01 KE
1. Kidney & Electrolyte
2. Renal Mechanisms
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Urinary Acidification by Proximal Straight Tubules

Previous Serial Number: NHLI-68

Principal Investigators: David Warnock, M.D.
Maurice Burg, M. D.

Other Investigators: Gerald Vurek, Ph.D.

Cooperating Unit: Laboratory of Technical Development/NHLI

Project Description:

Objectives: The $CO_2$/bicarbonate buffer system plays a central role in the maintenance of physiologic acid-base balance. Bicarbonate ion is the principal urinary buffer. Most bicarbonate is reabsorbed by the proximal tubule. In order to elucidate the mechanisms of urinary acidification, we have attempted to:

1. develop the micro-methods to measure acidification of the renal tubule fluid;

2. define the kinetics of generation and maintainance of transepithelial bicarbonate gradients in isolated proximal straight tubules.

Methods:

1. The isolation and perfusion of tubule segments from the rabbit kidney has been described previously. Superficial and juxtamedullary proximal straight tubules were distinguished on morphological and anatomical grounds.

2. A microcalorimetric method was developed to measure total $CO_2$ content of small fluid samples. This was done in collaboration with Dr. Vurek of the Laboratory of Technical Development, NHLI.

3. Cation/anion permeability ratios were determined using the Goldman-Hodgkin-Katz equation for the analysis of dilution and bionic potentials across the tubule wall.

4. A first-order kinetic model has been developed to describe the transepithelial movement of total $CO_2$ across the proximal straight tubule. Solution of the initial condition problem provides pump and leak rate constants, as well as a steady-state level of $CO_2$ in the individual tubule. The total $CO_2$ content of the luminal fluid is satisfactorily described as a function of the transit time of the perfusate. The transit time of the luminal fluid is varied by changing the perfusion rate.

Major Findings:

1. When the total $CO_2$ concentration in the perfusate and bath is 27.5 mM, proximal straight tubules reabsorb $CO_2$ causing the concentration in the lumen to decrease. When the concentration of total $CO_2$ in the perfusate is zero and that in the bath is 27.5, $CO_2$ enters the lumen, causing an increase in concentration. At slow flow rates a steady state concentration of $CO_2$ is reached in the lumen. The steady state level differs between tubule populations. In proximal straight tubules from superficial nephrons the mean steady-state luminal total $CO_2$ content is 16 mM, while in proximal straight tubules from juxtamedullary nephrons it is 9 mM.

2. It is the "leakiness" of the tubule epithelium to $CO_2$ that accounts for this difference. Both populations of tubules have similar pump rates, but the juxtamedullary tubules have a smaller leak of $CO_2$ back into the tubule lumen than do the superficial tubules. The kinetic model predicts the lower steady-state $CO_2$ level that results from this difference in leak rates.

3. The lower leak rate in the juxtamedullary tubules is consistent with other observations of relative cation/anion permeability ratios. On the basis of dilution and biionic potentials the tubules from juxtamedullary nephrons are calculated to be less leaky to anions than are those from superficial nephrons.

4. The effects on $CO_2$ of acetazolamide (which inhibits carbonic anhydrase) was determined. Acetazolamide caused the steady state level of luminal $CO_2$ content to increase in straight tubules from both superficial and juxtamedullary nephrons. It was not possible to distinguish, however, whether the pump or leak process was affected.

Proposed Course of Project:

1. The agreement between bicarbonate permeability calculated from the electrical measurements and the total $CO_2$ permeability measured directly suggest that the $CO_2$ leaks across the epithel-

ium as a bicarbonate ion. Further studies are necessary to confirm this point.

2. We will develop a pH glass microelectrode. The measurement of pH is necessary to distinguish dissolved $CO_2$ from the bicarbonate anion both of which contribute to the total $CO_2$. It is necessary to make this distinction in order to find out whether bicarbonate ions are transported per se or whether the primary mechanism is hydrogen ion transport.

3. These studies will be extended to other segments of the rabbit nephron.

Keyword Descriptors: Proximal Tubule, $CO_2$/Bicarbonate, Acidification, Picapnotherm

Honors & Awards: None

Publications:

Warnock, D.G., Burg, M.B., Vurek, G.G.: Urinary acidification by proximal straight tubules. Kidney International 6:110A, 1975 (Abstract, paper presented to the 7th Annual Meeting of the Society of Nephrology. Washington, D. C., 1974).

Vurek, G.G., Warnock, D.G., Corsey, R.: Measurement of picomole amounts of carbon dioxide by calorimetry. Analytical Chemistry. 47:765-767, 1975.

Project No. Z01 HL 01207-01 KE
1. Kidney & Electrolyte
2. Renal Mechanisms
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Glucose transport in the Proximal Convoluted Tubule

Previous Serial Number: None

Principal Investigators: David Warnock, M.D.
Maurice Burg, M.D.

Other Investigators: Clifford Patlak, Ph.D.

Cooperating Units: Theoretical Statistics and Mathematics Branch, NIMH

Project Description:

Objectives: It has been shown that in rabbits glucose transport in the proximal convoluted tubule is related to the generation of a transepithelial potential difference and the reabsorption of salt and water. The lumen glucose concentration is rapidly lowered in the proximal convoluted tubule, although in rabbits there is a relatively high permeability of the tubule epithelium to glucose. The result of the high permeability is a significant influx of glucose into the lumen down the length of the proximal convoluted tubule. We have quantified the contribution made b this passive influx to the total glucose transport capability of the proximal convoluted tubule of rabbits.

Methods:

1. The functional aspects of glucose transport have been previously defined by this laboratory (Tune and Burg, Amer. J. Physiol 221:580-585, 1971). This previous work provided the following parameters of glucose transport in the rabbit proximal convoluted tubule; passive b th to lumen glucose permeability, maximal glucose transport rate and affinity of the transport process for glucose.

2. A system of linear, differential equations were developed in collaboration with Dr. Patlak of the Theoretical Statistics and Mathematics Branch of the NIMH. This system described the removal of glucose from the tubule lumen by active transport and bulk flow, and the passive entry of glucose into the lumen by transepithelial diffusion. The equations were numerically integrated with the MLAB language of the DEC-10 computer facilities available at the NIH.

Major Findings:

1. A significant load of glucose is presented to the proximal convoluted tubule by transepithelial passive glucose influx. At physiological flow rates and glucose concentrations, nearly half of the glucose load originates in the passive transepithelial influx. Therefore, the glucose from the bath is as important as that of the original perfusate in any processes related to the active transport of glucose. We conclude that the passive entry of glucose ("leaked load") could be a significant factor in the reabsorption of salt and water from rabbit proximal tubules.

2. At physiologic flow rates and glucose concentrations, it is unlikely that solvent drag accounts for more than 2% of the glucose removed from the luminal compartment.

3. The lumen glucose concentration rapidly falls to a steady-state level within the first 2 millimeters of tubule length. The steady-state level is achieved when the active efflux rate equals the rate of passive glucose influx. The steady-state level is typically 0.5 mM when the initial perfusate and bath glucose concentrations are 5.5 mM. The lumen glucose present in the steady-state originates exclusively from the passive glucose influx from the bath.

Proposed Course of the Project: The analysis is essentially complete, and provides a means of analyzing the significance of the passive influx component of glucose and other solutes.

Keyword Descriptors: Proximal Tubule, Solute Back Flux

Honors & Awards: None

Publications: None

Project No. ZO1 HL 01208-02 KE
1. Kidney & Electrolyte
2. Renal Mechanisms
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Mechanism of salt and water transport by proximal renal tubules.

Previous Serial Number: NHLI-71

Principal Investigators: Maurice B. Burg, M. D.
Nordica Green

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: The proximal nephron reabsorbs approximately 50% of the glomerular filtrate. The mechanisms by which this occurs have been only partially characterized. There is a considerable body of evidence from micropuncture and microperfusion studies suggesting that sodium is actively reabsorbed providing the primary driving force for fluid and salt transport. The process is complicated, however, and apparently involves coupling between the transport of sodium and various other solutes as well as coupling to cellular metabolism as a source of energy. The purpose of these continuing studies is to investigate the interrelationships.

Methods: The rabbit isolated perfused proximal convoluted tubule preparation developed in this laboratory and described in previous reports was used to analyze the transport processes.

Major Findings:

1. Previously we found that sugar (glucose) or amino acid (alanine) in the perfusate caused a voltage oriented negative in the lumen of the proximal convoluted tubule and also caused a small, but significant, increase in the rate of fluid absorption. It was conceivable that the sugar and amino acid are metabolized by the epithelial cells energyzing the sodium pump which drives fluid transport. Alternatively, the sugar and amino acid might be co-transported with sodium, as in the intestine, accounting for the effect. In order to distinguish between these possibilities we tested the effects of a sugar (α-methyl-D-glucoside) and an amino acid (cycloleucine) known to be transported but not

metabolized, by proximal tubule cells. When placed in the perfusate, either α-methyl-D-glucoside or cycloleucine caused the rate of fluid absorption and the voltage to increase, suggesting that the transport of sugars and amino acids rather than their metabolism is coupled to salt and fluid transport. Additional evidence for this view was provided by the effect of phlorizin which specifically inhibits glucose transport. A low concentration ($10^{-5}$ M) of phlorizin in the perfusate caused the rate of fluid absorption and the voltage to decrease.

2. When glucose and/or alanine was added to the perfusate there was a striking change in the appearance of the epithelial cells. They swelled, protruding into the lumen. The change most likely is due to entry of glucose and alanine into the tubule cells during transport of non-electrolytes. Because of the additional solute in the cells, water enters by osmosis, causing the cells to swell.

3. Although active sodium transport is generally believed to be the basis of fluid absorption in this segment, the evidence has been inconclusive. There are numerous other theories, including suggestions that there is no active sodium transport or that sodium and chloride are co-transported by a linked mechanism. In order to test further the importance of sodium transport for fluid absorption, sodium was entirely omitted from the perfusate and bath. When the sodium was replaced by choline, tetramethyl ammonium, or lithium, the rate of fluid absorption and the voltage fell to zero. In contrast, when chloride was omitted (replaced by nitrate), there was no change. Evidently, transport of sodium but not of chloride is essential for fluid absorption.

4. In most tissues the active transport of sodium is linked to that of potassium, and omission of potassium results in inhibition of the sodium transport. When potassium was omitted from the bath, the rate of fluid absorption and the voltage across the proximal tubules fell to zero, additional evidence that active sodium transport is primary and that it has a requirement for potassium similar to other tissues.

5. In earlier micropuncture studies it was found that omission of bicarbonate caused the rate of fluid absorption to decrease. Therefore, we tested the affect of bicarbonate on the isolated proximal convoluted tubules. Omission of bicarbonate from the perfusate and bath caused the rate of fluid absorption to decrease by approximately one-third, similar to the micropuncture results. Several theories have been advanced to explain this bicarbonate dependence. One theory emphasizes that there is a change in tubule fluid chloride and bicarbonate concentration as bicarbonate is reabsorbed. The chloride concentration in the

lumen increases and that bicarbonate decreases. Considering that bicarbonate permeates the tubule more slowly than chloride, it presumably has a higher reflection coefficient. Therefore, the concentration gradient for bicarbonate (whose concentration is higher in the bath than in the lumen) might cause fluid absorption by osmosis. We tested this theory by interchanging methyl sulfate (which permeates the epithelium slowly, as does bicarbonate) and chloride in the perfusate. The rate of fluid absorption did not change, suggesting that anion concentration differences are not important for fluid absorption. Another theory that purports to explain the dependence of fluid absorption on bicarbonate emphasizes the well known role of hydrogen ion secretion in bicarbonate reabsorption. Bicarbonate presumably is necessary for hydrogen secretion across the lumen border of the tubule cells. It has been proposed that the hydrogen ion secretion is coupled to sodium entry into the cells by an exchange process. In the absence of bicarbonate the hydrogen ion secretion would be reduced, limiting sodium transport. The theory implies reciprocal dependence of bicarbonate and sodium reabsorption. We intend to test for this by measuring the effect on bicarbonate reabsorption of removing sodium from the perfusate and bath.

6. The enzyme carbonic anhydrase is important for bicarbonate transport in proximal tubules. Therefore, we tested the effect of acetazolamide which is an inhibitor of carbonic anhydrase and is a mild diuretic. Acetazolamide ($10^{-5}$M) caused the rate of fluid absorption by proximal convoluted tubule to decrease approximately as much as did removal of bicarbonate. It has been proposed that acetazolamide has an action in proximal tubules in addition to inhibiting carbonic anhydrase, i.e. that it inhibits fluid absorption directly and independently of bicarbonate transport. We intend to test this theory by seeing whether acetazolamide causes a further decrease in the rate of fluid absorption in the tubules in bicarbonate-free solutions.

7. In the studies outlined thus far the conditions used were similar to those in the early proximal tubule in which the perfusate is an ultrafiltrate of serum. Under these conditions the rate of fluid absorption is apparently normal comparable to the rate found in vivo. As fluid traverses the proximal tubule, however, its composition changes. Organic solutes, such as sugars, amino acids, and lactate are reabsorbed, causing their concentrations to decrease markedly in the lumen. As noted above, when isolated proximal tubules are perfused with low concentrations of the organic solutes, the rate of fluid absorption is greatly reduced. The low rate of fluid absorption apparently is less than the normal rate in vivo under what seems to be similar conditions in the late proximal convoluted tubule. The reason for this difference is of interest since it might involve a previously unrecognized factor important for fluid absorption. One

possibility is that there is a hormone or substrate lacking in the in vitro experiments. In attempting to identify such a factor we have tested the effect of added mineralocorticoids, glucocorticoids, glutamine, and free fatty acids. The results were negative. There was no change in fluid absorption. We intend to continue these studies by testing the effect of additional hormones and substrates. Another possibility is that the anatomically more distal parts of the proximal convoluted tubule function differently from the earlier segment. We have not been able to identify the anatomical location ("early" vs. "late") of the proximal tubule fragments that we study. We assume that since the fragments are dissected at random, they include "late" as well as "early" convoluted tubules, but there is no proof of this. Therefore, we will attempt to identify and study "late" proximal convoluted tubules in order to see whether they function differently from the early part and do not require organic solutes in the perfusate for normal rates of fluid absorption.

Proposed Course of Study: In addition to the proposals listed above, we intend to test the effects of a number of diuretic drugs on the proximal tubules in order to discover whether they have any important effect on this segment and, if so, what the mechanism of the effect is.

Keyword Descriptors: Convoluted Tubules, Fluid Absorption, Voltage, Organic Solutes, Bicarbonate, Acetazolamide

Honors and Awards: None

Publications:

Burg, M.B.: The mechanism of fluid absorption by proximal convoluted tubules. VI International Congress of Nephrology, Florence, Italy, June 8, 1975.

Burg, M. B.: Two Chapters submitted for publication, The renal handling of sodium chloride and Mechanisms of action of diuretic drugs, The Kidney, Edited by Barry M. Brenner, M.D. and Floyd C. Rector, M.D., Published by W. B. Saunders Co.

Project No. Z01 HL 01209-08 KE
1. Kidney & Electrolyte
2. Renal Mechanisms
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Ion Transport in the Cortical Collecting Tubule

Previous Serial Number: NHLI 70

Principal Investigators: Larry C. Stoner, Ph.D
Maurice B. Burg, M.D.

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: We previously found that cortical collecting tubules, in addition to regulating water reabsorption in response to vasopressin, also actively reabsorb Na from and secrete K into the tubule fluid. In the present studies we are investigating the effect of diuretic agents on ion transport in this segment.

Methods: Are the same as previously reported.

Major Findings: We had previously reported that low concentrations of acetazolamide in the bath caused the transepithelial voltage to become more negative in the cortical collecting tubule. This was taken as evidence for the existence of a urinary acidification process. We proposed that acetazolamide inhibited hydrogen ion secretion, reducing the positive voltage caused by that process, and thus increasing the observed negative voltage.

In initial studies we have found that the same concentration of acetazolamide ($2X10^{-5}M$) has little or no effect on Na and K transport in the cortical collecting tubule. Higher concentrations ($2X10^{-4}M$ and $10^{-3}M$) also result in a transient increase in voltage which is followed by a reduction. The voltage decreases were 30% (n=5) and 50% (n=4) of the initial voltage at the two concentrations. The decrease in voltage occurs between 10 and 30 min. after administration of the drug and is not reversible when the

drug is removed. In addition, $10^{-3}$ of acetazolamide caused a small decrease (-22%; n=2) in the lumen to bath flux of $Na^{22}$ and an increase in the bath to lumen flux of $K^{42}$ (+25%; n=7). Since these changes in voltage and transport were observed only at high concentrations of the drug their relation to the in vivo diuretic effects of the drug are questionable.

Proposed Course of Project:

1. Additional experiments are needed testing the effect of the lower ($2X10^{-5}$ M) concentration of acetazolamide on transport of Na and K. In addition, the present studies were carried out without bicarbonate in the tubule lumen. It will be of interest to see how acetazolamide affects Na and K transport when bicarbonate is present in the perfusate.

2. Since chlorothiazide is believed to exert its diuretic action in the distal nephron, its effect on the cortical collecting tubule will also be studied.

Keyword Descriptors: Collecting Tubule, Ion Transport, Diuretics

Honors and Awards: None

Publications: None

Project No. Z01 HL 01210-01 KE
1. Kidney & Electrolyte
2. Renal Mechanisms
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30,1975

Project Title: Mechanism of salt transport by isolated segments of amphibian distal nephron

Previous Serial Numbers: NHLI 69

Principal Investigators: Larry C. Stoner, Ph.D.

Other Investigators: David Hinton, Ph.D.

Cooperating Units: None

Project Description:

In the amphibian distal nephron as much as 60% of the NaCl that is filtered at the glomerulus is reabsorbed, diluting the urine. Previous investigators reported lumen negative transepithelial voltages in amphibian distal tubules but did not report lumen positive voltages, such as we previously found in the early mammalian distal tubule (thick ascending limb of Henle's loop).

Methods: Are identical to those previously described for perfusing isolated renal tubules in vitro.

Major Findings: We observed that the "distal convolution" of the amphibian nephron contains at least two functionally distinct segments: The early segment exhibits a lumen positive voltage (observed in 3 species - frog, toad and salamander) and absorbs NaCl from the lumen at a high rate. Since chloride moves out of the lumen against an electrochemical gradient, it is actively transported. In these properties as well as in the effects of diuretic drugs this segment is similar to the mammalian thick ascending limb of Henle's loop. Since the amphibia lack loops of Henle we have named this segment the "diluting segment." The second segment is the late distal tubule. It exhibits a lumen negative voltage and absorbs sodium at about one-fourth the rate of the diluting segment.

Continuing the study of the amphibian distal nephron, we have now measured the permeability to water of the diluting segment, the late distal tubule and the collecting ducts (salamander). In all three segments the permeability to water was not measurably different from zero. Further, the water permeability was not increased by the antidiuretic hormone (ADH), arginine vasotocin. Thus, in this species of amphibia, the late distal nephron differs from that in the mammal where ADH dramatically increases the water permeability.

We have also used the electronmicroscope to ascertain that the diluting segment and the late amphibian distal tubule differ morphologically. We found that the cells of the diluting segment are morphologically similar to those of the mammalian thick ascending limb, and those in the late distal tubule are similar to their mammalian counterpart - the late distal convoluted tubule or early cortical collecting tubule. This morphological distinction had not previously been made.

Proposed Course of Project: Completed.

Keyword Descriptors: Chloride transport, Amphibian nephron, Urinary dilution

Honors and Awards: None

Publications: Stoner, L. Isolated segments of the amphibian distal nephron: The diluting segment. Manuscript in preparation.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Anion transport across individual amphibian erythrocytes

Previous Serial Number: None

Principal Investigators: Larry C. Stoner, Ph.D.
Floyd M. Kregenow, M. D.

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: To a large degree, our knowledge of ion transport in the red blood cell has served as a basis for understanding ion transport in other tissues. The electrical potential, ion gradients, and permeability to ions are parameters that must be determined to characterize ion transport. In red cells the methods used for measurements of electrical parameters and anion transport have been indirect and lacked precision. The purpose of the present studies is to develop precise and direct methods for measuring voltage, electrical conductance, and permeability to ions across single red cells. Such methodology should provide information valuable for understanding transport in erythrocytes and eventually in other cells as well.

Methods: Individual amphibian red blood cells are held snugly in a constriction in a specially prepared glass pipet. In this position the cell is a cylinder with hemispherical ends. A second glass pipet is centered within the pipet that holds the cell. Either of two types of inner pipets is used: 1) a glass microelectrode ("Ling-Gerard" type) which punctures the cell membrane for measurement of the cellular voltage and resistance, or 2) a larger pipet which does not enter the red cell, but is used to wash one end of the cell within the holding pipet with a radioisotope containing solution. Permeability to the isotope is determined from the amount of radioactivity that penetrates through the cell and appears in the external bath.

Major Findings:

1. Electrical Measurements

The intracellular voltage averaged -17 mv (negative in the cell) with pH 7. in the bath. Although this voltage is the same as that previously found by others, we feel the present technique is superior. We measured stable potentials that lasted 30 seconds or more, whereas using the previous techniques the voltages decayed within a few milliseconds after the puncture. Other laboratories have reported that variation of the extracellular pH leads to alteration of the red cell chloride concentration and subsequently the intracellular voltage. The present study confirmed this relationship. At pH 6.5 the observed cell voltage was -10 mv and at pH -8.1 the voltage was -26 mv.

Once a stable voltage was obtained, we passed small electric currents into the red cell to measure the specific resistance of its membrane. The results of 29 such attempts provided a mean value of 100 $\Omega \cdot cm^2$ (conductance of .01 $\Omega^{-1}$ $cm^{-2}$). In 80 other cells the same current was passed through the entire cell, lodged in the constriction of the holding pipet. In this experiment the current passed through the cell membrane twice in series, once on each side of the cell, since the electrode was not inserted into the cell. The resistance measured was exactly twice that found when the electrode was inside the cell. Thus, the specific membrane resistance calculated from the two experiments was identical, increasing the confidence in both results. Further, the agreement of the results indicates that the entire current passed through the cell in the second experiment and that the leak of current (and ions) around the cell in the constriction was negligibly small.

2. Radioisotope flux:

When $Cl^{36}$ is placed on one side of the cell within the holding pipet, it passes through the cell and appears in the bath. The measured chloride$^{36}$ flux is 0.84 X $10^{-6}$ $cm^{-2}$ min, equivalent to a partial chloride conductance of .05 $\Omega^{-1}$ $cm^{-2}$. This exceeds the total electrical conductance (G) of .01 $\Omega^{-1}$ $cm^{-2}$ by a factor of five. Therefore, the cell of $Cl^{36}$ movement is not electrically active i.e. it cannot be explained by simple passive diffusion. Two other observations support this concentration.

a. When a voltage (200 mv) is imposed across the cell during a flux measurement, $Cl^{36}$ flux increases, but the increase is much smaller than that theoretically pre-

dicted if all of the isotope flux were electrically active. The observed change in flux was used to calculate chloride conductance. The partial chloride conductance was half of the total electrical conductance and only one-tenth as great as the Cl conductance that would be calculated from the isotope flux in the absence of imposed voltage.

b. Replacement of 90% of the chloride on the bath side of the cell with PAH results in depression of the $Cl^{36}$ flux from the opposite surface (mean decrease 40%, n=7 cells). This indicates that the chloride fluxes in the two directions are linked. The results are consistent with the generally accepted hypothesis that chloride penetrates the red cell membrane by a "carrier mediated" mechanism.

Proposed Course of Project:

1. To evaluate the contribution of other ions to the electrical conductance.

2. To investigate further the mechanism of anion transport across the red blood cell.

Keyword Descriptors: Anion Transport, Erythrocytes, Membrane Resistance

Honors and Awards: None

Publications: None

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Volume regulation in nucleated erythrocytes

Previous Serial Number: None

Principal Investigators: Floyd M. Kregenow, M.D.
Larry C. Stoner, Ph.D.

Other Investigators: None

Cooperating Units: None

Project Description:

Previous studies from this laboratory have shown that avian erythrocytes contain a "volume controlling mechanism" which can regulate cell size in isotonic or anisotonic media. This mechanism returns cells to their original volume in either hypo, hyper, or isotonic media, even when the cation pump is blocked. In isotonic media, the response is hormone dependent, requiring one of the catecholamines. The mechanism allows cells to dynamically control their volume, a property which is critical to most animal cells. The process involves the controlled movement of considerable quantities of potassium into or out of the cell, accompanied by diffusable anion, primarily chloride, and osmotically obliged water. Conceptually, the mechanism consists of a receptor, transmitter, and effector. The latter is presumably located in the membrane and consists of transport mechanisms which would have been previously categorized as part of the leak. Previous evidence indicated that two transport mechanisms were involved: one operates when cells correct volume by losing cations (cell shrinkage), while the other functions when cells correct volume by gaining cations (cell enlargement).

Objectives:

I. To provide additional evidence that the transport process associated with cell enlargement is functionally separate from both cation pump and the transport process associated with cell shrinkage.

II. To determine the role anions play in both transport processes.

III. To determine whether the formation of functional "reconstituted ghosts" involves the volume controlling mechanism.

IV. To develop methods and procedures for measuring transport in a single cell.

Major Findings:

I. To provide further evidence that the transport process associated with cell enlargement is functionally separate from the classical pump and also different from the transport process responsible for cell shrinkage, we studied the response of cells modified in two ways. The first method involved treating normal cells with 1 mM furosemide. In the second, we replaced cellular Cl with $SO_4$, producing cells we have labelled "$SO_4$ cells." Both groups of cells display normal Na and K transport through the classical cation pump. In contrast, the process of cell enlargement with its net uptake of Na, K and $H_2O$ as well as the characteristic rapid bidirectional exchange of Na and K is as the characteristic rapid bidirectional exchange of Na and K is completely inhibited. Although both groups of treated cells can no longer control their volume by enlarging, the process of cell shrinkage remains intact. The phenomenon of shrinkage in cells treated with furosemide is unaltered, i.e. the loss of K, Cl and $H_2O$ and the characteristic increase in K efflux remain the same. The shrinkage phenomenon also remains intact in $SO_4$ cells, although the rate of electrolyte and water loss is 4 times slower.

II. Continuing our study of the role anions play in the process of cell enlargement and shrinkage (see Annual REport 1974), we tested whether any major change in cellular phosphate occurred during either phenomenom. Removing phosphate from the bathing medium had no effect on either process; nor is the total organic phosphate content of a TCA extract of cells ($37 \pm 2$ mM $PO_4/L_{onc}$ - 90% of which is ATP and phytic acid) altered during either process.

Sits, a disulfonic acid derivative, is an amino reactive agent which in human erythrocytes is known to inhibit anion transport without affecting cation transport. In duck erythrocytes this agent also inhibits anion transport, as indicated by a 99.7% reduction of $SO_4$ influx into "$SO_4$ cells," while it is without effect on the cation fluxes characteristic of cell enlargement and shrinkage.

Two media have been developed which will allow us to study the effects of pH on the one hand and pH and bicarbonate concentration on the other.

III. Human red cells can reacquire their normal cation impermeability after undergoing hemolysis in hypotonic media. The process of restoration requires changes in temperature and

electrolyte concentration and produces an altered cell form (called a ghost) which may have both a normal biconcave shape and cation content. One can also make ghosts from nucleated cells. To examine whether the process of restoration in these cells results in a predictable adjustment in cell volume, we examined ghosts which had lost 3/4 of their intracellular hemoglobin.

If the restoring solution differs in electrolyte content by as much as 100 mM KCl, so that the resultant ghosts have a 2-fold difference in KCl content, ghost volume (measured as the non-inulin space) remains the same ∿3%. This volume is similar to what one would predict if during restoration the ghosts, despite their different electrolyte content, reacquired the same $H_2O$ content of normal cells but were minus the volume normally occupied by 3/4 of the hemoglobin, (25% of the original cell volume). Ca must be present at the time of hemolysis for adequate restoration, and the process is also facilitated by the presence of $PO_4$.

Whether this phenomenon represents a form of volume regulation which is independent of intracellular protein content must await more precise measurements of ghost volume and neterogeneity using a Coulter cell volume analyzer. It should be mentioned that the volume controlling mechanism, mentioned previously, may not be involved.

Ghosts prepared in this manner can maintain a 20-fold concentration gradient for K, have a K permeability comparable to normal intact cells, but fail to respond to hypotonicity, hypertonicity, or norepinephrine. Thus, the volume controlling mechanism as defined in intact cells, is either inoperable or not present in these reconstructed ghosts.

IV. Procedures have been developed using nucleated red cells from the giant salamander, Amphiumia for measuring transport in a single cell preparation. Values obtained from this technique are being correlated with measurements of ion transport on a large cell population. (See individual project report of Stoner and Kregenow

Proposed Course of Project:

1. To continue the objective stated in II - IV.

2. To design experiments to test for the presence of the mechanism in human erythrocytes.

Keyword Descriptors: Cation Transport, Volume Controlling Mechanism, Cell Enlargement, Single Cell Measurements

Honors and Awards: Symposium talk in honor of Dr. M. Jacobs presented at 1974 Federation Proceedings and entitled, "Characterization of a Mechanism Capable of Regulating Cell Size in Nucleated Erythrocytes."

Publications: Effect of Norepinephrine and Hypertonicity on K Influx and Cyclic AMP in Duck Red Cells. Floyd M. Kregenow, Dianne E. Robbie, and Jack Orloff. Submitted to Am. J. Physiology for publication.

Two manuscripts in preparation.

Project No. Z01 HL 01213-05 KE
1. Kidney & Electrolyte
2. Experimental Cardiovascular Diseases
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Cardioglobulin B-s of human serum

Previous Serial Number: NHLI-67

Principal Investigators: Stephen Hajdu, M.D.
Edward J. Leonard, M.D.

Other Investigators: None

Cooperating Units: Biology Branch, NCI

Project Description:

Objectives: Cardioglobulin is the name we have applied to a mammalian protein system which regulates calcium entry into cells. We reported previously that 3 components of the system, cardioglobulin-A, -B and -C circulate in the blood plasma. Cardioglobulin-C contains bound calcium, cardioglobulin-A has a high energy phosphate. The cardioglobulin proteins in the blood become activated when cardioglobulin-B interacts with a cell surface component. The integrated action of the whole system is to release protein-bound cardioglobulin-C calcium. We believe that the protein system is located in a region immediately external to the plasma membrane and is capable of maintaining a local calcium ion concentration which is higher than and independent of the serum calcium. In muscle, the calcium of the cardioglobulin space (immediately external to the plasma membrane and in the T system) enters the cell during depolarization and causes shortening of the contractile protein. Cardioglobulin may be localized on the surface of other types of cells as well as muscle. Abnormalities in serum cardioglobulin activity have been found in human diseases, notably systemic lupus erythematosus.

Cardioglobulin is assayed on the isolated frog heart. Although the frog does not have the complete cardioglobulin system its cardiac muscle has a surface component on which mammalian cardioglobulin proteins can be assembled and activated. Activation of the system results in entry of calcium ions into the heart muscle, the reflection of which is increased contractile force. At high cardioglobulin concentrations, contracture of the heart muscle occurs.

In our previous work we used rat serum as a source of some cardioglobulin proteins and human serum for others. We now report the results of a study on human cardioglobulin. In this report we show that human serum cardioglobulin comprises 4 or possibly 5 components. The action of the whole system can be broken down into 3 separate steps. Step 1 requires the interaction of cardioglobulin -$B_1$ and -A. Step 2 requires cardioglobulin -$B_2$ and -A. The final step is mediated by cardioglobulin -C and -A.

Methods: An outline of protein chemistry used for the separation of the individual members of the system was given in a previous report (NHLI-67).

Major Findings: We found previously that the action of cardioglobulin on the frog heart could be divided into 2 steps. First, diluted human serum was equilibrated with the heart for 20 minutes at 25°C. During this time cardioglobulin -B became bound to the heart and was not removed by subsequent washing with Boyle-Conway solution. The heart after washing was called a B- heart. In the second step, addition of cardioglobulin-A and -C caused the cardiotonic action of the cardioglobulin system.

Our initial attempt to purify cardioglobulin-B was to separate serum into 2 fractions with 15% sodium sulfate. No -B activity was found in either the 0-15% supernatant solution or in the precipitate, but activity was recovered when the 2 fractions were combined. This showed that -B activity required 2 serum components which we called -$B_1$ and -$B_2$. The next advance in cardioglobulin -B fractionation was based on our finding that dextran sulfate (DS)precipitates one of the -B's. These findings suggested it might be possible to purify one of the -B components with DS. Therefore 500,000 MW DS was added to serum, in a final concentration of 400 µg/ml. The precipitate was separated by centrifugation and dissolved with 500 µg/ml protamine sulfate. We then determined whether this material could reconstitute B activity when combined with the serum sodium sulfate fractions $B_1$ or $B_2$. We found that DS precipitate produced B activity when mixed with -$B_1$ (15% sodium sulfate supernatant solution) but not with -$B_2$. Therefore DS precipitated -$B_2$. To test the supernatant of DS-treated serum for -$B_1$ activity, we first removed residual free DS by precipitating the serum proteins with 20% sodium sulfate. When we tested the DS (0-20) fraction for -$B_1$ activity by combin- with DS $B_2$, no -$B_1$ activity was found. This could occur if $B_1$ itself was inactivated by DS or if besides $B_1$ and $B_2$ a third component was required for the compilation of the B-reaction and it is this which is DS sensitive. As third component of the B- reaction, we thought of cardioglobulin -A, since it was known to be DS sensitive. We tested this latter possibility with a 23% sodium sulfate supernatant which lacks all the cardioglobulin components except ca 50% cardioglobulin A. Test-

ing of this fraction showed that it could reconstitute B activity when combined with DS-$B_2$ and DS (0-20). We concluded from these experiments that 3 components were required to make a B-heart: B1, found in a 20% sodium sulfate precipitate, and unaffected by DS; B2, a euglobulin, precipitable with DS; and a component inactivated by DS and found in the supernatant of a 23% sodium sulfate fraction of whole serum, which is probably -A. Experiments were made to determine whether all 3 of the B components had to be present simultaneously. It was found that a B heart was made when B1 + A, and in a separate step B2 + A was added to the heart. Reverse order or omission of A in either step led to negative results.

A-Inhibitor. We have shown that the cardioglobulin reaction occurs in a sequence of 3 steps: +B1 + A; B2 + A; C + A. Since all these components are present in serum, one would expect the complete sequence to occur when serum is added to the frog heart. However, addition of as much as 1.0 ml of human serum at 25°C, does not result in the characteristic action of cardioglobulin on the frog heart. Much less than 1.0 ml of serum (0.1-0.2 ml) causes contracture if the serum is applied in 2 steps, with a 10 minute Boyle-Conway solution wash in between the 2 steps. We asked whether serum after a 20 minute equilibration with a fresh frog heart at 25°C had A + C activity as tested on a B-heart. This used serum not only did not cause contracture of a B-heart, but it could even inhibit the action of fresh serum on a B-heart. It was shown that the addition of cardioglobulin-A, but not -C overcame the inhibition of the used serum. We concluded that equilibration of fresh human serum with a frog heart caused elaboration of an inhibitor of cardioglobulin-A which prevented the cardioglobulin reaction from going to completion.

We finally succeeded in purifying and storing human cardioglobulin components under the following conditions:

-A free of -C and -B2
-C free of -A and -B2
-B- free of -B2
-B2 free of -A, -C and -B1

Possession of these components enabled us to measure any cardioglobulin component of a human serum sample quantitatively.

Proposed Course of Project: This project comes to an end by September 30, 1975, due to the retirement of one of us (S.H.). In the time remaining an attempt will be made to assess the individual components of cardioglobulin in normal subjects and in paritents with systemic lupus erythematosus.

Keyword Descriptors: Lupus Erythamotosus, Calcium Transport

Honors and Awards: None

Publications: None

ANNUAL REPORT OF THE
LABORATORY OF TECHNICAL DEVELOPMENT
NATIONAL HEART AND LUNG INSTITUTE
JULY 1, 1974 THROUGH JUNE 30, 1975

This laboratory continues to develop new instruments and methods to facilitate medical research, diagnosis or therapy. In many respects, the boundaries of our knowledge and our ability to apply what we know are limited by our technology. Methods development and new instrumentation serves to widen our horizons and to convert knowledge to practice. We have a group of scientists selected for their interests and capabilities to identify requirements of biomedical science that can benefit from the application of instrumentation science and technology. We develop working systems that can be applied at N.I.H. in conjunction with ongoing research activities make the results available to the scientific community and industry by publishing the method or instrument. The areas of our activity include analytical methods and separation techniques, not only for chemicals but also for living cells; clinical applications include regional blood flow measurement, materials and methods development for better cardiopulmonary support for both isolated organs and whole animals.

An instrument for the rapid determination of the oxygen dissociation curve (ODC) of hemoglobin has been constructed and is presently operating in the Laboratory of Molecular Hematology. The instrument was developed in cooperation with scientists at the University of Milan primarily for the investigation of the fundamental properties of the interaction of hemoglobin, oxygen, and small effector molecules. It is interesting to note that physical theories based on the action of purified hemoglobin have been shown to be invalid from experiments done on sickle cell patients with varying degrees of irreversibly sickled cells (ISC's).

Fast, reliable thermistors developed for use in the study of biochemical reactions have now been thoroughly tested and two types are now available commerically. A new differential amplifier developed to be used with them has been incorporated into prototypes of two new instruments. One, a differential thermal titration calorimeter is to be used in conjunction with a new differential pH meter, to measure simultaneously the thermometric and potentiometric titration curve of a protein. The complete curve, from pH 4 to 10 can be run in 2 minutes on a 2 ml sample containing 0.1 μ mole of protein. Such titrations permit the protein chemist to identify the groups active in biochemical reactions as well as those reacting with inhibitors.

The second instrument is the stopped-flow calorimeter which operates in the millisecond range. With a resolving time of 3 millisecond it supplements the normal stopped-flow spectrophtometer permitting observation of pre-steady-state reactions that lack an observable optical absorption. The reaction of glutamic dehydrogenase and ATPase are typical samples. The exploitation of this system will be carried out on several well documented reactions to demonstrate its advantages. In another development a high-

speed quenched flow apparatus has been constructed and thoroughly tested. A stepping motor 4 syringe drive accurately controlled as to rate and distance of advance is used with three ball mixers and an automatic sampling valve. For example, in the study of sacroplasmic reticulum at the Institute of Aging, ATP is added to the SR's in the first mixer, at different times $CA^{+}$ is added in the second mixer, and again at different times TCA is added to quench the reaction and then the sample is quickly collected by the automatic sampler. A resolving time of 3 milliseconds has been achieved.

Calorimetry using heat production as an indicator of biochemical and metabolic activity may be useful in indicating changes in individual cells without harm to the cells or modifying their behavior.

In the host of cytological tests for specific biochemical defects, immunoresponses of various leucocytes, and transformation and oncogenesis induced by mitogens, antigens, specific factors and organisms. Specific cell response requires a destructive or at best intrusive procedure such as staining or assay of radiothymidine uptake by radioautography. A method whereby the heat given off by individual cells can be observed as interference fringes around each cell in the microscope field requires only minor modification of the microscope optics and a substage thermal cycling device to alternately condense and remove a liquid film from the underside of a thin film supporting the specimen. Granulocytes ingesting bacteria show their heat production is related to the number of organisms ingested and on autolysis. The system should permit the same cells to be used as controls and the proportion of cells responding to various agents determined with the test cells available for further culture or other assays.

A cell growth assay system explored the use of specific spatial filtering opportunities afforded by the availability of coherent laser light sources and led to the development of a prototype stem cell colony assay device using a moving grid to selectively modulate scattered light from stem cell colonies and discriminate against scattered light background from the semisolid medium. A laboratory model for evaluation is being produced on a Department of Defense contract to N.C.I.

An optical device made possible by the availability of laser sources is able to indicate the velocity spectrum of moving blood corpuscles in a capillary bed and small vessels by analysis of the optical frequency shift produced by scattering from the moving cells. Observation of the capillary bed of fingertips, and exposed internal organs while vasoactive maneuvers and pharmocologic agents are administered indicates that the system can analyze vascular responses from the frequency spectrum of backscattered laser light. Comparison with xenon washout techniques in a clinical test indicated weaknesses in conventional methods and the desirability of the new method for non-intrusive skin measurements and relatively small needle probes or light pipes for internal organs.

Non-invasive non-destructive nuclear magnetic resonance methods for circulation studies is now ready for some trials on clinical materials and large animals with the acquisition of a large magnet at the Medical College of Wisconsin on our contract with them for studies using electronic systems developed here and reduced to practice there.

Systems of measuring blood gasses on a continuing basis for acute care and vital support have been developed in conjunction with methods for providing extracorporeal gas exchange. As these values change rapidly, a fast responding continuous indicator is desirable without continuous consumption of blood for analysis. Following techniques developed previously for ultramicro determination of picomolar amounts of $CO_2$ and Bicarbonate in kidney tubule micropuncture samples, current systems sample by introducing a micro diffusion cell into the vascular system at the end of a catheter and sample the gasses without withdrawing blood. The galvanic reduction current is used for oxygen and the heat of reaction with lithium hydroxide indicates $CO_2$ with adequate precision and accuracy. Present work is directed to dependable diffusion sampling beads.

Some separations of biochemical interest require the use of phases with low interfacial tension. When low interfacial tension phases are used in our centrifugal countercurrent chromatograph emulsification and loss of stationary phase degrades the system. A $30^{o}$ rotor was constructed to reduce emulsification forces and the system tested with several extraction phases with a range of interfacial tensions to see the effect of the angle rotor. The single rotor performed well with even extremely low interfacial tension polymer phases and was demonstrated to be able to separate bone marow colony stimulating factor at high efficiency thus contributing to CSF work and validating the concept of the angle rotor countercurrent chromatograph which in addition to the above has advantages over other conventional liquid chromatographic systems.

Centrifugal separation systems using counterrotating columns to avoid rotating seals while permitting continuous flow have been unsuitable for blood separation due to the secondary centrifugal force set up by the counterrotation. A new concept in continuous flow to rotating systems published recently provided the basis for a new blood separating centrifuge that eliminates the rotating seal that is generally conceded to be a source of cell damage, microemboli and generally incompatible with blood due to the limits imposed by materials suitable for rotating seals. Current work uses the doubled back loop in which the rotor speed is twice the speed of the loop which is guided by a tooth belt driven guide. Blood continuously introduced is separated into plasma and cells as in the plasmaphoresis (celltrifuge) machine. Platelet survival in the new device indicated minimum damage. Tests with our sheep perfusion heart preservation system also indicated great advantages could be expected to result from the modification. Applicability to plasmaphoresis, cell and platelet transfusions and organ preservation is anticipated. It would also appear to be a desirable approach to washing frozen preserved blood cells.

In order to advance a method or technique, it is often necessary to show how it can solve certain problems of interest. In the case of luminescence spectroscopy, involving either fluorescence or phosphorescence, there are many biochemical problems amenable to the method. We choose to work on some intrinsically interesting problems to show the utility of luminescence.

A phosphorescence study was performed which showed that $Ag^+$ markedly enhances the phosphorescence of tryptophan (3-fold) and totally quenches the fluorescence; the study also showed that proteins phosphorescence was altered by $Ag^+$ in various ways. The effect of $Ag^+$ in proteins containing sulfhydryl groups could be attributed to total luminescence quenching by energy transfer to $Ag^+$-mercaptide absorption bands. However, only fluorescence is quenched in non-sulfhydryl proteins. It was found that 10% methanol snows at 77°K were suitable matrices for the study, and it was suggested that previous studies on protein phosphorescence done in glasses of organic solvents may have studied only denatured proteins. Enzyme activity measurements showed no denaturation on 10% methanol.

The study of membranes and lipid micelles by fluorescence has become popular in the biochemical literature. We have found that certain dyes (TNS, ethidium bromide, quinacrine) show markedly altered fluorescence in detergent solutions of different concentration. In fact, the critical micelle concentration (cmc) can be detected by following such probe fluorescence. We have measured the emission of some detergent solutions in the presence of various amounts of salt (which alters the cmc) and confirmed the effect.

The binding of fluorescent compounds by certain enzymes has yielded information about the active sites. L. Brand reported that Auramine O was bound by liver alcohol dehydrogenase but not by yeast alcohol dehydrogenase as shown by the marked enhancement of Auramine O fluorescence. On the other hand, we have examined the quenching of protein fluorescence of these enzymes by Auramine O, and find that both enzymes do bind the dye. In contrast to previous reports, therefore, dyes whose fluorescence is not enhanced cannot be assumed not to bind to a given protein. Similarly, the antimalarial drug primaquine was reported by T. Li to inhibit liver alcohol dehydrogenase noncompetitively but not to inhibit yeast alcohol dehydrogenase. We find that both enzymes bind the drug, confirm that yeast ADH is not inhibited, but find that the inhibition is competitive with the coenzyme, NAD.

These studies have produced results of interest to biochemists and again illustrate the utility of fluorescence and phosphorescence methods.

The section on Pulmonary and Cardiac Assist Devices has continued studies to imporve the inherent safey of extracorporeal pulmonary support perfusion, particularly as related to long-term use.

We have developed a method of coating the blood contacting surfaces of our membrane oxygenators with a pure silicone rubber gum rather than the usual

silica reinforced material previously used. Perfusions performed in sheep have shown better platelet sparing, reduced heparin requirement and reduced general morbidity. Recent clinical experience elsewhere using pure gum surface oxygenators appears to confirm our experience of greater blood compatability.

The ability to fabricate multilayer membranes now permits the production of membranes with desirable surface properties and specific permselectivities. A membrane incorporating carbon black did not produce the usual transient agranulocytosis universally found at the beginning of bypass with all other membrane lungs and renal dialysis systems.

We have recently acquired capability to cast fluorosilicone gum membranes as well as to coat exisiting membranes with polymers other than silicones.

For use in long term bypass we have explored several methods to measure cardiac output. Almost all presently used techniques provide intermittent data on cardiac output, the accuracy of which is often dubious. The membrane lung in the extracorporeal blood circuit serves as a meter by providing a known quantity of oxygen to allow continuous determination of cardiac output in all patients undergoing right sided bypass with the membrane lung, using the technique of Fick cardiac output determination. Provided all blood from the membrane lung is returned into the root of the aorta, left sided cardiac output determinations are similarly feasible.

We have further explored factors to successful preservation of the mammalian heart, using sheep hearts. The perfusion circuit was coated with pure gum silicone rubber by a technique developed here. We have previously shown that sheep hearts when perfused ex vivo at 10 and 13$^{o}$C continued to show electrical activity and exhibited forceful ventricular contractions for many days. In this study we sought to determine which of the cellular fractions of whole blood were critical to success of organ preservation. Separation of fresh whole blood into several fractions was accomplihsed in the "Celltrifuge". We studied platelet-poor plasma, platelet-rich plasma, platelet-rich plasma with whole blood added to give a hematocrit of 1-2%, and incompletely separated plasma, i.e. plasma containing some red blood cells, white blood cells, and platelet-rich plasma (hematocrit 1-2%). All studies were performed at 13$^{o}$C. As with hearts perfused with whole blood, hearts in all four groups showed ventricular contractions for various lengths of time, with a peak left ventricular systolic pressure as high as 70 mm Hg. and a heart rate of 15-20 contractions per minute. However, the longest and most forceful contractions were in groups having some red blood cells in the perfusate, and particularly the group where red blood cells were only incompletely separated. These hearts on rewarming had no weight gain, and had excellent ventricular function on rewarming. Of particular interest in our study with a sheep heart is the finding that continuous ventricular contraction at 13$^{o}$C is an excellent criteria of cardiac viability. Furthermore, that in spite of $PO_2$ levels above 100 mm Hg, ventricular contractions ceased if flow of fresh perfusate fell below

15 cc/min. This observation may suggest a different mechanisms to cardiac failure secondary to impaired coronary circulation: "ischemia" need not only be the result of hypoxia, but may also be a manifestation of inadequacy of as yet undefined substrates. These substrates appear to be of molecular weight 5,000 to 50,000 as determined from our previous studies using blood ultrafiltrate as perfusates.

Project No. Z01 HL 01401-02 LTD
1. Laboratory of Technical Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 thorugh June 30 1975

Project Title: Angle Rotor Countercurrent Chromatography

Previous Serial Number: NHLI-80

Principal Investigator: Yoichiro Ito

Other Investigator: Robert L. Bowman

Cooperating Units: None

Project Description:

Objectives: Demonstration of the potential capability of the $30^{o}$ angle rotor countercurrent chromatograph in terms of the following:

1. Applicability of two-phase solvent systems having varieties of interfacial tension and viscosity.

2. Determination of optimum operational ranges in revolutional speeds and flow rates.

3. Comparison to high pressure chromatographic method.

Methods Employed and Major Findings:

The $30^{o}$ angle rotor previously described was employed throughout. The coiled helix column was first filled with the stationary phase and sample solution introduced through the feed line. Then the apparatus was spun at a constant speed while the mobile phase was pumped through the feed line using a syringe driver. The eluates were either monitored directly through a u.v. monitor or fractionated into test tubes for further analysis.

1. Applicability of two-phase solvent systems:

Several two-phase solvent systems were selected in consideration of their varieties in physical property and versatility in solute partitioning. Using these solvent systems, performance of the apparatus was evaluated on separation of biological compounds as follows:

a. High interfacial tension phase system: Ethylacetate/10% acetic acid, 5%NaCl (1:1) was used for separation of 5 catecholamine metabolites.



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Using the same solvent system abnormal levels of VMA and HVA were also detected from 100 microliter of urine sample.

b. Medium interfacial tension phase system: N-BuOH/1M potassium phosphate (pH 6.5) (1:1) was used for separation of 5 purines and pyrimidines using the organic phase as a mobile phase. Also a similar phase system of n-BuOH/0.1M ammonium formate (1:1) was used for separation of 7 dipeptides all containing tyrosine moiety by applying a linear gradient of dichloroacetic acid between 1% and 0%.

c. Low interfacial tension phase systems: Chloroform/acetic acid/ 0.1N HCl (2:2:1) was used for separation of 9 DNP aminoacids and sec-BuOH/0.3% dichloroacetic acid (1:1) for partition of bovine insulin.

d. Polymer phase system (extremely low interfacial tension and high viscosity): A polymer phase system composed of 6% charged polyethylene glycol 6000, 6% dextran T 500, and 0.05M sodium phosphate buffer was used for separation of bone marrow colony stimulating factor with pH gradient elution.

In spite of the diversity of their physical properties, all above solvent systems showed satisfactory phase retention and yielded a high efficiency separation with elution times ranging from 5 to 18 hours. The results also indicated that either organic or aqueous phase can be used as a mobile phase, and that the gradient elution technique was adaptable as in the conventional liquid chromatography.

2. Determination of optimum operational ranges of revolutional speeds and flow rates:

Using the chloroform/acetic acid/0.1N HCl (2:2:1) system, separations of 9 DNP aminoacids were performed by applying combinations of revolutional speeds (500, 700, 1000, and 1200 rpm) and flow rates (0.25, 0.5, 1.0 and 1.5 ml/hr) with a 0.3 mm i.d. coiled helix column. All combinations applied showed satisfactory resolution for all 9 peaks. Although the highest resolution was achieved at 500 rpm at 0.25 ml/hr of flow rate by an overnight run, a good separation was also obtained at 1200 rpm at 1.5 ml/hr within 6 hours. Thus the results indicate that the present system permits a wide range of operational conditions.

3. Comparison to high pressure liquid chromatography:

Twelve isomers of monohydroxybenzo(a) pyrene (1 through 12-HOBP) have previously been resolved into 2 groups by a high pressure liquid chromatographic technique. It used a gradient elution with methanol solution 35% to 75% through a column packed with hydrocarbon-coated beads. The first peak consisted of 2, 6, 8, 9-HOBP, and the second peak, 1,3,4,5,7,10,11, and 12-HOBP. Changing the solvent system or

gradient pattern has failed to improve the resolution.

Attempts were made to separate these samples with the $30^{o}$ angle rotor using a 0.3 mm i.d., 70 m long coiled helix column. Using hexane/55% methanol (1:1), or hexane/methanol/0.01M sodium phosphate (pH8) (100:55:45), 8 isomers (1,2,3,4,5,6,8, and 9 - HOBP) were resolved distinctly into 3 peaks, the first peak consisting of 2,8, and 9-HOBP, the second 1 and 7-HOBP and the third, 3,4, and 5-HOBP. With the former phase system, the first peak appeared to be further resolved into two peaks, probably 2 and 8-HOBP and 9-HOBP. Using a single solute, column efficiency was estimated to be over 15000 theoretical plates. Although the present method failed to resolve all components, it yielded much higher resolving power than a refined high pressure liquid chromatographic method.

Significance to Biomedical Research and the Program of the Institute:

The present method, in addition to eliminating complication from the solid supports, proposes the following advantages over other conventional liquid chromatographic methods. Applicability of two-phase solvent systems is almost universal and covers high interfacial tension hexane/$H_2O$ systems to an extremely low interfacial tension polymer phase systems that are hardly applicable to the conventional chromatography. The efficiency yielded with a fine analytical column exceeds 15000 theoretical plates while that in the conventional liquid chromatography is limited to several thousand theoretical plates. The method can be easily scaled up for preparative separation using a large bore column. The technique is highly reproducible and simple enough for unskilled technicians to follow. On the basis of these advantages, we feel that the method will be very useful in biomedical and biochemical research.

Proposed Course:

Although the performance of the present prototype is satisfactory for our laboratory use, it shows loss of lubricant from the bearings which necessitates weekly lubrication. Also the revolutional speed is limited to 1200 rpm because of the present design in that the lower end of the column holder projecting a few inches below the lower bearing level tends to overload the bearing and damage the aluminum column holder. These deficiencies should be eliminated in the future model.

Keyword Descriptors:

Angle Rotor, two-phase solvent systems, separation of biological compounds, Catecholamine metabolites, VMA, HVA, purines and pyrimidines, separation of 7 dipeptides, separation of 9 DNP aminoacids, partition of bovine insulin, polymer phase system, and colony stimulating factor.

Honors and Awards: None

Publications:

1. Ito, Y., Hurst, R. E., Bowman, R. L., and Achter, E.K.: Countercurrent Chromatography, Separation and Purification Methods, (1), 133-165 (1974).

2. Ito, Y., and Bowman, R. L.: Angle rotor countercurrent chromatography, Analytical Biochemistry, in press.

Project No. Z01 HL 01402-01 LTD
1. Laboratory of Technical Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30 1975

Project Title: New Flow-Through Centrifuge Without Rotating Seals.

Previous Serial Number: None

Principal Investigators: Yoichiro Ito and Jacques Suaudeau

Other Investigators: Theodor Kolobow, Gerald G. Vurek, and Robert L. Bowman.

Cooperating Units: None

Project Description:

Objectives: 1. Designing and constructing a flow-through centrifuge based on the principle reported by Adams.

2. Investigation of potential capability of the centrifuge.

Methods Employed and Major Findings:

a. Principle:

Consider a horizontally placed bowl with a flexible tube connected at the center of the lower surface. The other end of the tube is then supported above the bowl on its central axis, making a loop of the tube. When the bowl rotates at an angular velocity of 2 w around the vertical axis and the loop simultaneously revolves around the same axis at w, the tube continuously untwists and remains always untwisted. In this situation the tube spontaneously counterrotates around its own axis at - w during operation.

b. Design of the centrifuge:

The prototype has been constructed by modifying a conventional refrigerated centrifuge. The frame of the centrifuge head consists of three parallel horizontal rectangular plates ridgedly linked and driven by the motor shaft as a unit. The frame holds a centrifuge bowl at the center, a contershaft on one side and a tube supporting a tubular guide on the opposite side, all vertically mounted in ball bearings. A stationary pulley mounted on the motor housing on the axis of the centrifuge is coupled through a toothed belt to an identical pulley

mounted on the countershaft to counter-rotate the shaft with respect to the rotating frame. This motion is further conveyed to the centrifuge bowl by 1:1 gearing between the countershaft and the bowl. This arrangement doubles the angular velocity of the centrifuge bowl to accommodate the principle outlined above. To support the counter-rotating flow tubes the tubular guide is actively counter-rotated at -w by means of a pair of 1:1 ratio toothed pulleys coupled to the hollow shaft and the countershaft.

A doughnut shaped silicone rubber bag (800 ml capacity) equipped with three flow lines is fitted inside the centrifuge bowl. The transparent lucite cover enables observation of its contents under stroboscopic illumination. Three silicone rubber flow tubes from the rubber bag are led down through the center of the centrifuge bowl, then upward through the guide tube, and finally through the center hole of the stationary centrifuge cover. When properly balanced, the centrifuge bowl can be rotated at up to 2000 rpm without detrimental vibration.

c. Application to plasmapheresis:

In order to demonstrate the capability of the centrifuge, sheep blood was introduced into the centrifuge directly from the animal, while effluents of plasma and red blood cells were returned, after sampling, to the animal. Flow rates through the individual lines were controlled by two rotary pumps, one set on the whole blood line and the other on the plasma line, the thrid line having flow equal to the difference between the two pumps. With a constant feed rate of 60 ml/min , plasma free of red blood cells was harvested at 12 ml/min at 1000 rpm or 18 ml/min at 1300 rpm. During 12 hours of continuous flow of plasma at 18 ml/min, blood and plasma samples were collected by intervals to study changes in platelet counts. The results showed a 50% reduction in blood platelet count within the first one hour, and a reduction to 30% of baseline values by the 12th hour of operation. These results are similar to reported studies using a membrane lung in a similar perfusion system without centrifuge and are much superior to flow-through centrifuge that uses rotating seals.

Significance to Biomedical Research and the Program of the Institute:

The conventional flow-through centrifuge utilizes rotation seals which can become a source of leaks between inflow and outflow lines and represents a weak point in the machinary, in terms of duration, complexity and fragility of the pieces, and necessity for a continuous and equal lubrication. When those continuous flow centrifuges are adapted for an inline blood separation, as realized for collection of

blood cells, rotating seals become critical in terms of platelet injury, red cell hemolysis, obstruction of the channels and of the lubrication grooves by aggregates, and following inefficiency of blood separation. The present device eliminates all these complications and therefore will contribute to biomedical research where separation of vulnerable cells and intracellular particulates is involved. Thus the method may be useful in cell washing and elutriation, zonal centrifugation and countercurrent chromatography.

Proposed Course:

1. Refinement of the present prototype to improve protection of the flow tubes at the bottom of the centrifuge bowl and at the center of the centrifuge cover.

2. Further investigation on plasmapheresis.

3. Construction of the second prototype which is equipped with an interchangeable centrifuge bowl so that various configurations of the bowl can be conveniently tested for cell washing, elutriation and countercurrent chromatography.

Keyword Descriptors:

Flow-through centrifuge without rotating seals,Separation of vulnerable cells and intracellular particulates, cell washing, elutriation, zonal centrifugation, and countercurrent chromatography.

Honors and Awards: None

Publications: None

Project No. Z01 HL 01403-02 LTD
1. Laboratory of Technical Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30 1975

Project Title: Eye Motion Measurement by Ultrasound

Previous Serial Number: NHLI-84

Principal Investigator: Frank W. Noble

Other Investigators: Robert L. Bowman

Cooperating Units: None

Project Description:

Objectives: Deviant motion of the eye at the first several harmonics of the heart rate may be indicative of circulatory problems within the head and neck areas. There is some evidence that variation from normal motion may warn of impending stroke.

Major Findings: Since an acoustic wave travelling in air is totally reflected by any liquid or solid surface, it is practical to interrogate a small region of the front surface of the eye by means of ultrasound. The phase delay between separate transmitting and receiving transducers is modulated by the eye motion. This delay is converted to amplitude modulation and recorded, resulting in a record of eye motion vs. time.

Various methods of mounting the transducers with respect to the eye of human subjects have been investigated in an effort to reduce artifacts due to extraneous motions of the subject's head. The associated electronics have been improved, incorporating crystal frequency control, narrow band receiving equipment, and heavy amplitude limiting. Cross-correlation equipment has been employed to compare the eye motion with the patient's electrocardiogram to discriminate against signals which are asynchronous with the heart.

Significance to Biomedical Research and the Program of the Institute: If deviant motions of the front surface of the eye are found to correlate with circulatory pathology, the significance could be considerable.

Proposed Course: Interference problems produced by random motions of the head and eye with respect to the transducers are of such magnitude that even cross-correlation with the ECG produces only a barely recognizable signal at the heart rate.

A much better method of transducer mounting and signal processing must be devised in order to make the system practical.

Keyword Descriptors: Eye Motion, Stroke Prediction, Ultrasonics, and Circulation.

Honors and Awards: None

Publications: None

Project No. Z01 HL 01404-07 LTD
1. Laboratory of Technical Development
2. Section on Pulmonary & Cardiac Assist Devices
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Membrane Lung Systems for Long Term Support

Previous Serial No.: NHLI-82

Principal Investigator: Theodor Kolobow

Other Investigators: Edward Stool, Paul Weathersby, Joseph Pierce, Robert L. Bowman, and Jacques Suaudeau

Cooperating Units: Armed Forces Radiobiology Research Institute, and Section on Lab Animal Medicine and Surgery

Objectives:

1. Current heart lung machines cannot be safely used clinically in excess of 4-6 hours. We are demonstrating the safety of the membrane lung in long term use of many days and weeks duration.

2. Defects in membranes, resulting in leaks from pinholes, has been the major stumbling block to routine clinical use of the membrane lung. We have determined the cause of these pinhole defects, and applied a new technique to overcome this problem.

3. Blood compatibility of synthetic materials is the most important, if not exclusive, limiting factor to routine, safe use of artificial internal organs. Because of large surface areas involved, this limitation applies particularly to the membrane lung, and to a lesser degree, they also apply to the artificial kidney, heart valves, heart assist devices, and other implantable organs and devices. The various factors that contribute to polymer blood compatibility are not clear, and very likely include a variety of aspects. It has been shown that "impurities" normally present in synthetic polymers do at times contribute to enhanced blood compatibility, and at other times to a lowered blood compatibility. Because of high gas transmission we have concentrated our investigation on silicone rubber membranes of different purities, and to charged membranes.

4. We are investigating the physiologic range of hypothermic temperature, and the type of perfusate, for isolated sheep heart preservation.

These studies arise from the knowledge of present day limitations in preserving hearts beyond 24 hours without functional impairment on reimplantation.

Methods Employed and Major Findings:

1. The Membrane Lung

Membrane casting technology has been significantly enhanced to allow multilayer casting of gas permeable membranes optimally suited for specific applications. As an example, up to five separate discrete layers of polymer have been cast with presently available casting machine, with a membrane overall thickness of 50 micrometers. It is now possible to custom design, within limits, a membrane lung most suitable for specific applications: conventional heart-lung machine, long term respiratory assist, selective enrichment of blood with oxygen, or preferential removal of carbon dioxide. With application of special high efficiency membranes, the rating of the spiral membrane lung has been raised, under standard conditions, to 80-100 $cc/(m^2)(min)$ for oxygen and carbon dioxide. This transfer is 2-3 times greater than for other stationary membrane lung models. The spiral membrane lung as of now is suitable both for gravity bypass, as well as for pump-through bypass.

a. Standard Membrane (3 layer technique)

As presently used, this membrane has exceptionally strong burst strength, and a blood compatibility consistent with "Medical Grade" silicone rubber. Artificial lungs using this type of membrane represent the state of the art for commercial medical membrane lung devices.

b. Pure Silicone Gum Membrane (3 layer technique)

Significantly improved blood compatibility is seen when no silica filler is added to silicone rubber. Because of the casting technique employed, the burst strength of this new membrane is nearly equal to the standard membrane.

c. "Charged" Membrane (three layer technique)

The blood contacting surface contains added acetylene black and pure silicone gum rubber.

d. "Charged" Membrane with Pure Gum Interface (four layer Technique)

The charged layer is physically below a smooth layer of pure silicone gum rubber.

e. Charged Membranes Coated with Fluorosilicone Gum Rubber (four layer technique)

The thin coating adjacent to blood consists of fluorosilicone gum rubber.

f. Silicone Gum Rubber Membrane, Coated with Hypothrombogenic and Nonthrombogenic Polymer Systems (5 layer technique)

Perfluorinated ethyl cellulose, and diethylaminoethyl cellulose (radiation grafted with heparin) can be readily applied to charged membranes, with good adherence. Similarly, because of the physical nature of the "charged" membranes, many other organic coatings can be applied to these surfaces (such as some hydrogels), and tested for use in the membrane lung.

A. Clinical Applications:

We believe a great number of patients who ultimately require treatment with the membrane lung for blood respiratory gas exchange represent complications of intensive respiratory care. The patient population at the Clinical Center is in addition burdened by research patients in whom therapeutic interventions have rendered them immunologically susceptible to pathogen organism invasion.

We have provided extracorporeal respiratory gas exchange with a membrane lung in a young boy with leukemia, immunosuppression with total body irradiation followed by bone marrow transplantation, for 12 days. Technically, bypass was accomplished in a routine manner, without complications. The patient did succumb to the underlying disease process, however.

B. Animal Research:

Arteriovenous shunt of blood across the membrane lung without the use of a blood pump.

The performance of new membranes developed and fabricated in this laboratory is presently undergoing animal testing. In this study, the common carotic artery and the external jugular vein were cannulated and connected through silicone gum rubber coated silicone rubber tubing across the test membrane lung. Heparin was administered at the time of cannulation, but none was given further the moment the arteriovenous shunt was opened. We measured changes in blood flow resistance across the membrane lung, changes in platelet and white blood cell count, and white blood cell differential counts.

1. A control series of membrane lungs made of standard "Medical Grade" silicone rubber membrane uniformly shows a prompt fall in platelet count, white blood cell count, and an agranulocytosis during the first 10 minutes, and a gradually rising perfusion pressure.

2. Membrane lungs made of silicone gum rubber show no changes in platelet count, and no rise in perfusion pressure for duration of the heparin effect (6 hours).

3. Membrane lungs made with charged silicone gum rubber surfaces are unique as there is no change in platelet count, or white blood cell count at any time. Unique to this class of membranes, the platelet sparing effect persists for a much longer time compared to any previous membrane studied, or reported. Thus, 65% of platelet count remains in peripheral blood, compared to 35% with gum silicone rubber membrane, and 20% with standard "Medical Grade" silicone rubber membrane after 3rd day without heparin.

Studies with other membranes and membrane coatings produced by us are under way.

4. Studies reported above were all done on lambs, which are notoriously more difficult to work with in terms of the coagulation system compared to man.

The pure silicone gum membrane developed by us was applied to man in just one single case. In this instance, no change in platelet count, white blood cell count and differential, or a rise in perfusion pressure were observed; virtually no heparin was given during the two day perfusion.

The improved results in sheep with new membranes and membrane coatings developed by us leads us to believe that even greater improvement can be expected when applied to man. At the same time, the sheep remains an excellent model to find often minute differences among candidate membrane materials.

We feel that removal of silica filler and processing aides was beneficial to improve blood compatibility. The addition of acetylene black into a membrane system appears to exert an additional blood cellular sparing effect. Our results show that our technical capability of producing pinhole free silicone membranes has allowed us to use a material generally considered inferior for commercial use, and to produce a blood contacting surface with improved blood compatibility.

Our findings represent the first practical improvement in silicone membrane technology for use in the membrane lung.

C. Membrane Lung Priming:

We have found it critical to remove all gas from the membrane lung to reveal the ultimate potential of a candidate membrane. Blood platelets typically adhere and aggregate around gas bubbles, thus initiating a sequence of events leading to thrombosis.

In our newer system, all priming is done under vacuum, where all air is removed except for water vapor. Priming with degassed prime is then accomplished under hydrostatic pressure.

Unique to this system is speed of priming: once degassed, the membrane lung is primed and ready for use in less than five minutes. In addition, this technique is superior to the carbon ioxice prime technique because of speed and a gurantee that no gas bubbles remain any place within the membrane lung.

2. Ex Vivo organ preservation by hypothermic perfusion.

Dog and sheep hearts perfused with a synthetic medium at 5-10$^{o}$C after 24 hours of perfusion uniformly become edematous, with a continuous rise of their vascular resistances. These hearts during preservation remain flaccid, without electrical or mechanical activity.

We have since shown that sheep hearts continuously perfused at 10$^{o}$C and above with fresh donor blood can maintain electrical and mechanical activity, there was no edema and upon rewarming, the hearts had good ventricular function. We have also shown that a flow of at least 15 cc/min of whole fresh blood was necessary to assure organ viability. Similarly, an ultrafiltrate enriched solution of fresh blood (MW cutoff 50.000) at a rate above 20 cc/min supplied sufficient factors to assure a continuously contracting heart, and excellent cardiac preservation.

Blood being an excellent perfusate, this study was designed to determine which of the cellular fractions (if any) of whole blood were critical to success of organ preservation.

Sheep hearts were excised and treated in the same manner as before. The perfusate consisted of various plasma fractions from an adult sheep, separated from blood in a flow through centrifuge (celltrifuge).

The various perfusates were as follows:

1. Platelet poor plasma.

2. Platelet rich plasma.

3. Platelet rich plasma with whole blood added to give a hematocrit of 1-2%.

4. Incompletely separated plasma, i.e. plasma containing some red blood cells, white blood cells, and platelet rich plasma (Hct 1-2%)

All studies were performed at 13°C.

As with hearts perfused with whole blood, the hearts in all four groups showed ventricular contractions for various lengths of time, with a peak left ventricular systolic pressure as high as 70 mm Hg, and a heart rate of 15-20 contractions/minute. However, the longest and most forceful contractions were in groups having some red blood cells in the perfusate, and particularly the group where red blood cells were only incompletely separated. These hearts on rewarming had no weight gain, and had excellent ventricular function on rewarming.

Significance to Biomedical Research and the Program of the Institute:

Since 1970, about 20 patients have been saved worldwide with the membrane lung from acute respiratory failure unresponsive to conventional treatment using various types of membrane lungs.

The complexity of long term membrane lung support places unusual requirements on blood damage and membrane lung performance. We have shown that silica free silicone rubber membrane has superior blood compatibility compared to commercially used medical grade silicone rubber membrane containing silica fillers, and processing aides; a much lower red blood cell lethal and sublethal damage, and much improved compatibility to blood platelets.

We have shown that addition of carbon to the membrane matrix in a membrane lung significantly reduces damage to blood cellular elements. In particular, platelet change is nonexistent when heparin is used, and platelet count remained a high 65% of baseline values after three days in the absence of any heparin. It is likely that heparin use during long term or short term bypass in man could be severely curtailed if not eliminated when these novel membranes are used. Similarly, these new surfaces if applied to other artificial internal organs (artificial booster heart, total artificial heart, heart valves, the artificial kidney, vascular grafts, etc.) could similarly impart a new dimension of safety and reduce patient morbidity.

Our work on sheep heart preservation suggests the importance of certain blood cellular fractions to successful organ preservation. It is evident that lessons learned have great relevance to other internal organ preservation.

Proposed Course:

1. Studies will be continued to assess use of various silicone gum rubber (including fluorosilicone gum) and additives.

   The effects of carbon concentration, location, and surface coating will be similarly investigated.

2. A program will be initiated to develop a novel type of membrane lung suitable for long term implantation. This will include the preparation of new types of membranes, and new surfaces, and new designs, to make a reliable, compact unit.

3. The merit of using a membrane lung in acute respiratory care will be assessed by blood carbon dioxide exchange alone to the exclusion of oxygen, in an arteriovenous shunt without blood pumping. The simplicity of this procedure is appealing as it requires low blood flows, and no blood pump. It is hoped that this technique will lower the high incidence of brochopleural fistula in the treatment of acute respiratory failure, by reducing the minute ventilatory volumes, and peak inspiratory pressures. Similarly, it may be found useful in the treatment of some states of hypercapnea.

4. We have the capability of producing ultrathin (5 microns), non-reinforced or reinforced silicone rubber membrane, which could be used as a human burn dressing. A pilot laboratory study will be initiated to explore this possibility.

Keyword Descriptors:

Artificial lung, membrane lung, permselective membranes, silicone rubber membranes, silicone gum rubber membranes, gas exchange, respiratory assist, blood compatibility, arteriovenous shunt, organ perfusion, heart preservation, and ex vivo perfusion.

Honors and Awards: None

Publications:

1. Kolobow, T., Hayano, F., and Weathersby, P. K.: Dispersion-casting thin and ultrathin fabric-reinforced silicone rubber membrane for use in the membrane lung, J. Assn. Adv. Med. Instrum., in press.

2. Weathersby, P. K., Kolobow, T., and Stool, E. W.: Relative thrombogenicity of polydimethylsiloxane and silicone rubber constituents, J. Biomed. Materials Research, in press.

3. Kolobow, T., Stool, E. W., Sacks, K. L., and Vurek, G. G.: Acute Respiratory Failure: Survival Following 10 Days Support with a Membrane Lung, J. Thoracic & Cardiovascular Surgery, in press.

Project No. Z01 HL 01405-02 LTD
1. Laboratory of Technical Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Analysis of Microcirculation by Coherent Light Scattering

Previous Serial Number: NHLI-85

Principal Investigator: Michael D. Stern, Donald L. Lappe

Other Investigators: Robert L. Bowman

Cooperating Units: Laboratory of Experimental Therapeutics, NHLI

Project Description:

Objectives: To continue exploratory development of a method of non-invasively measuring flow and other parameters of the microcirculation in tissues by means of analysis of the spectrum of coherent light doppler-scattered from the tissue. The measurement of these parameters in extremities is of prime importance for the evaluation of peripheral vascular diseases, while the measurement in internal organs offers an important tool for the study of pharmacodynamics and vascular physiology. Specific objectives are to develop a prototype instrument and method, and demonstrate the feasibility of using this technique in the physiology laboratory and clinical setting.

Methods:

(a) Theoretical Analysis

We are engaged in an ongoing theoretical study of the kinetics of multiple scattering of coherent light in tissue in the presence of blood flow, with the goal of providing a framework for the interpretation of emperical spectra obtained in real physiologic settings, and relating these to the true distribution of blood flow velocities in the microvascular bed, and to the total tissue flow in regions of varying vascular geometry.

(b) Instrument Development

A prototype laser scattering apparatus capable of studying human skin and various tissues in experimental animals has been designed and built, together with the associated signal processing equipment. The instrumental requirements for more advanced modifications of the system, such as the use of fiber-optic light pipes to measure flow in inaccessible regions, are under development.

(c) Experimental

Experiments have been undertaken with the prototype apparatus to demonstrate the ability of this method to monitor the real-time dynamics of circulation in human skin and other tissues, to validate the correlation of the doppler spectrum with other measures of tissue blood flow, and to show the use of the laser doppler technique in studying regional flow in internal organs during pharmacologic interventions. A clinical protocol has been developed for the use of the instrument to monitor blood flow in the skin of patients with peripheral vascular diseases.

Major Findings:

1. Theoretical analysis indicates that under a broad class of circumstances the spectral shift of scattered light can be analyzed in terms of a model of random walk of photons through tissues, suffering repeated doppler shifts when scattered by red cells. With idealized assumptions this model can be analyzed analytically and predicts the general shape observed for spectra from actual tissues. The analysis predicts that the complete velocity distribution of blood flow in microvascular bed is, in principle, recoverable from the spectra, and that certain weighted average bandwidths of the spectra should be proportional to tissue blood flow, in fixed vascular geometry.

2. The prototype instrument has been designed to measure the spectra and the weighted bandwidths described above. An appropriate stable helium neon laser and photomultiplier have been procured, together with the necessary electronics for real time spectrum analysis and autocorrelation of the photodetector signal. With this system it has been possible to record spectra with good signal to noise ratios from human skin, and from the surface of internal organs (kidney, liver) of experimental animals. These spectra vary in the expected manner with interventions which produce vasomotor changes in the tissues, and with occlusion of vascular supply. A number of dynamic vascular reflexes associated with posture, emotion and respiratory pattern, and with thermoregulation are easily studied.

3. An experiment was undertaken in collaboration with the Department of Biomedical Engineering of the University of Washington to measure the correlation of our method of measuring the blood flow in the forearm with the $^{133}$Xe washout technique. Good correlation was shown; the ability of the laser instrument to monitor continuously made possible the study of the dynamics of the vascular bed at the site of injection of radioactive xenon, raising the possiblity of injection artifacts in the xenon method which could not be previously documented.

4. An experiment was undertaken to show the feasibility of using the laser instrument to monitor continuously microvascular flow in small regions of

exposed renal cortex in the rat. Good, reproducible spectra were obtained, which vanished when the renal artery was occluded. Response of the renal cortical perfusion to a number of vasoactive drugs (dopamine, angiotensin, norepinephrine, isoproterenol) was easily followed in time and quantitated, and steady state dose-response curves measured. At present this method appears very promising qualitatively and quantitatively; it remains to be calibrated and validated against other methods for regional organ blood-flow.

5. A clinical protocol has been established to use the prototype instrument to study skin microcirculation in patients with a variety of conditions during interventions covered by other NIH protocols. An experiment to study finger circulation in Raynaud's disease, with the use of nitroglycerin therapy is underway.

Significance of the Program to the Institute:

The diagnosis and followup of peripheral vascular diseases, the study of circulation in burns and grafts, and the study of tissue blood flow under dynamic conditions in response to pharmacologic and hemodynamic alterations are among the important applications forseen for the technique.

Proposed Course:

1. Continuation of theoretical analysis.

2. Demonstration of further applications of the method in physiology and clinical situations.

3. Experiments to further validate this method against other meausres of microcirculatory parameters.

4. Attempts to extract from the spectra more detailed information about the distribution of flow in the compartments of the microvasculature.

5. Planning for further advanced development of prototype instruments.

Keyword Descriptors: Microcirculation, capillaries, blood flow, laser, doppler effect, renal blood flow, peripheral vascular disease, tissue perfusion, light scattering, skin blood flow.

Honors and Awards: None

Publications: 1. Stern, M.: In vivo evaluation of the microcirculation by coherent light scattering. Nature 254: No. 5495, March 1975.

Project No. Z01 HL 01406-11 LTD
1. Laboratory of Technical Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Fluorescent Complexes of Proteins

Previous Serial Number: NHLI-79

Principal Investigator: Raymond F. Chen

Other Investigators: None

Project Description:

Objectives: Fluorescent labeling of proteins is an important area of fluorescence spectroscopic methods in biomedical applications, and is practiced in histochemistry as well as biophysical studies. We wish to find and characterize new dyes which may have novel properties, and to show how they may be applied.

Methods Employed: Dyes which are investigated may bind covalently or simply adsorb to a given protein. In the case of covalent binding, the dye is reacted with the protein, and unreacted dye is separated by passing the protein solution through a Sephadex column. The properties of the fluorescent conjugate are investigated by fluorescence spectroscopy and lifetime measurements. Dye-protein adsorbates are similarly studied under equilibrium conditions.

Major Findings: With Dr. Walter Stewart of NINDS we have measured the spectra and quantum yield of a number of compounds he has synthesized to label the proteins of nerve axons. The most promising of these compounds, tentatively named N-110, is a highly anionic dye with a quantum yield of about 0.2 in water which will replace Procion Yellow, currently used for such neurochemical studies.

Two related dyes, N-86 and N-105, were studied to see if they could be used to label proteins for biophysical studies such as depolarization of fluorescence. N-105 seems to be promising: it reacts readily with proteins, has a quantum yield near 0.2, and the lifetimes of the fluorescent conjugates are in the range of 11 nsec. Because of its highly negatively charged character, N-105 may be a good dye for labeling basic proteins such as histones.

Another class of dyes, quinacrine and quinacrine mustard, was studied. The latter, abbreviated QM, labels proteins and gives conjugates with

lifetimes ranging from 5 to 13 nsec. The dye has a $pK_a$ near 7.7 and may be useful in labeling ampholines, thus giving a built-in fluorescent pH indicator in isoelectric focusing experiments. The polarization, lifetimes, corrected spectra of quinacrine and QM conjugates were written up in a paper to be submitted shortly.

Bilirubin-albumin complexes are known to show light sensitivity. Recent reports in the literature suggest that the photosensitivity is due to photooxidation of bilirubin by singlet oxygen. The latter in turn is produced by bilirubin photosensitization. Thus,bilirubin catalyzes its own decomposition. A project has started showing that ascorbic acid and $\alpha$ -tocopherol may slow the process. This is in concord with literature reports that these antioxidants scavenge singlet oxygen. The rate of decomposition of bilirubin in these complexes is followed either by fluorescence or absorption spectra.

It is known that some enzymes are inhibited, or their response to activators altered, after reaction with various reagents. By reacting enzymes with fluorescent dyes, we can probe the active site or the control sites. The technique requires specific attachment of a dye to a given area on a protein. In preliminary experiments, we have shown that fluorescamine and quinacrine mustard produce inhibition of alcohol and glutamate dehydrogenases.

Significance to Biomedical Research and the Program of the Institute:

The use and characterization of new dyes for protein studies advances the state of the art of fluorescence spectroscopy, which in turn is a major tool in histochemistry and biochemistry. The work continues the institute's traditional leadership in the area of fluorescence.

Proposed Course:

There are several dyes which can combine covalently with amino groups, and which can serve as pH markers. Dyes such as quinacrine mustard, fluorescein isothiocyanate, and neutral red could be incorporated into an isoelectric focusing matrix to mark the pH. We hope to try this method out with various dyes. We also wish to study the dye distribution in protein conjugates; i.e., in a preparation where the protein has an average of 3 dyes bound per mole. How many molecules have 1, 2, 3, 4, 5, or 6 dyes attached? The answers will enhance our understanding of the accessibility of sites to fluorescent labeling and will show whether it is a purely random process or not.

Keyword Descriptors: Flucrescence, dyes, quinacrince, quinacrine mustard.

Honors and Awards: None

Publications: 1. Chen, R. F.: Fluorescent Enzyme Inhibitors, a table for Handbook of Biochemistry, Gerald Fasman, Ed., Chemical Rubber Co., in press.

Project No. Z01 HL 01407-12 LTD
1. Laboratory of Technical Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Applications of Fluorescence in Biochemistry

Previous Serial Number: NHLI-78

Principal Investigator: Raymond F. Chen

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: In order to advance a method or technique, it is often necessary to show how it can solve certain problems of interest. In the case of luminescence spectroscopy, involving either fluorescence or phosphorescence, there are many biochemical problems amenable to the method. We choose to work on some intrinsically interesting problems to show the utility of luminescence.

Methods Employed: We have previously acquired and modfied instrumentation which permits a wide range of measurements to be made. These include: phosphorescence and fluorescence excitation and emission spectra, lifetimes, quantum yields, and stopped flow kinetic measurements.

Major Findings:

1. A phosphorescence study was performed which showed that $Ag^+$ markedly enchances the phosphorescence of tryptophan (3-fold) and totally quenches the fluorescence; the study also showed that proteins phosphorescence was altered by $Ag^+$ in various ways. The effect of $Ag^+$ in proteins containing sulfhydryl groups could be attributed to total luminescence quenching by energy transfer to $Ag^+$-mercaptide absorption bands. However, only fluorescence is quenched in non-sulfhydryl proteins. It was found that 10% methanol snows at 77°K were suitable matrices for the study, and it was suggested that previous studies on protein phosphorescence done in glasses of organic solvents may have studied only denatured proteins. Enzyme activity measurements showed no denaturation in 10% methanol.

2. The study of membranes and lipid micelles by fluorescence has become popular in the biochemical literature. We have found that certain dyes (TNS, ethidium bromide, quinacrine) show markedly altered fluorescence in

detergent solutions of different concentration. In fact, the critical micelle concentration (cmc) can be detected by following such probe fluorescence. We have measured the emission of some detergent solutions in the presence of various amounts of salt (which alters the cmc) and confirmed the effect.

3. The binding of fluorescent compounds by certain enzymes has yielded information about the active sites. L. Brand reported that Auramine O was bound by liver alcohol dehydrogenase but not by yeast alcohol dehydrogenase as shown by the marked enhancement of Auramine O fluorescence. On the other hand, we have examined the quenching of protein fluorescence of these enzymes by Auramine O, and find that both enzymes do bind the dye. In contrast to previous reports, therefore, dyes whose fluorescence is not enhanced cannot be assumed not to bind to a given protein. Similarly, the antimalarial drug primaquine was reported by T. Li to inhibit liver alcohol dehydrogenase noncompetitively but not to inhibit yeast alcohol dehydrogenase. We find that both enzymes bind the durg, confirm that yeast ADH is not inhibited, but find that the inhibition is competitive with the coenzyme, NAD.

Significance to Biomedical Research and the Program of the Institute:

These studies have produced results of interest to biochemists and again illustrate the utility of fluorescence and phosphorescence methods.

Proposed Course: The work on the alcohol dehydrogenases will be completed, and combined with measurements made on the ORTEC nanosecond spectrometer.

Several problems reamain in the use of fluorescent probes to follow micellization, as the curves of fluorescence vs. detergent concentration show several inflections indicative of detergent structure not shown by other methods. Further investigations into this phenomenon are planned.

The stopped-flow fluorescence device will be used to follow some reactions where $D_2O$ exchanges with protons in the intermediate time range. If successful, this would be the first use of stopped-flow fluorescence to follow hydrogen exchange.

Key Descriptors: Phosphorescence, micelles, dyes, alcohol dehydrogenase.

Honors and Awards: None

Publications: 1. Chen, R. F.: Phosphorescence of Tryptophan and Proteins in the Presence of Silver Ion, Arch. Biochem. Biophys. 166, 584, (1975).

Project No. Z01 HL 01408-10 LTD
1. Laboratory of Technical Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Methodology in Fluorescence Measurements

Previous Serial No.: NHLI-77

Principal Investigator: Raymond F. Chen

Other Investigators: None

Cooperative Units: None

Objectives: To advance the state of the art in methods and instrumentation used in fluorescence spectroscopy, a technique which is popular in biomedical sciences, but which has many facets.

Methods Employed: As in the past, we have tested commercially available apparatus to see what modifications of hardware or methods are required to obtain data desired in biophysical chemistry. Many instruments are designed to work in a particular way, but when actually in use by a biochemist, it is often found that the desired experiment requires some modification of the apparatus.

Major Findings:

1. The ORTEC 9200 nanosecond spectrometer was designed to investigate fluorescence decay kinetics. However, it cannot in itself determine lifetimes, which are one of the main objectives desired. To analyze the decay curves, we have had the ORTEC connected so that the digital data representing the decay curves are read into the teletype and saved on paper tape, or read onto magnetic tape. The data then are analyzed by convolution; i.e., one convolutes the lamp flash with theoretical decays representing certain lifetimes and then compares the experimental and theoretical decay curves to get the lifetimes. This is conveniently done through our terminal connections to the PDP-10 computer of the computer center. Alternatively, one can use "lifetime standards", consisting of partially quenched quinine solutions and compare decay curves to see what the actual lifetime is. We have also obtained a Schoeffel GM 100 monochromator and had a flange made to allow emission to be analyzed. By obtaining decay curves at different emission wavelengths, it will be possible to obtain spectra of the emission at different times after the exciting flash.

2. We tested a prototype Aminco corrected spectra spectrofluorometer, which was designed primarily to give corrected excitation and emission spectra. The instrument was loaned to us for 2 months. We compared excitation

and absorption spectra, which should coincide; also we compared the instrument's emission spectra with those we obtained on another Aminco-Bowman spectrofluorometer and manually corrected. Generally, the instrument was satisfactory, but areas needing simplification were pointed out to the designers. In particular, the choice of time constants and scanning rates was too limited, and the need for different phototubes for excitation and emission spectra was criticized. At present, a phototube has been found combining low noise and the desired spectral response characteristics has been found. We also made phosphorescence measurements on the instrument and showed that this could be done conveniently. No other commercial instrument having correction abilities operates on DC detector system, so that we demonstrated that this instrument is the only one able to obtain corrected phosphorescence excitation and emission spectra. Some of these spectra will be incorporated into a paper on the effect of denaturation on the phosphorescence of proteins.

3. We have been interested in the use of metal ions as probes of protein structure because, when metal ions interact with tryptophan and tyrosine, they may alter the fluorescence and phosphorescence. Work done with the phosphorescence of tryptophan and proteins in the presence of the heavy metal ions, $Ag^{+}$, and $Hg^{++}$ were found to be facilitated by the use of aqueous snows rather than to attempt to make clear glasses. A paper summarizing past work showing fluorescence quenching by these ions was prepared and included recent work showing phosphorescence enhancement due to increased intersystem crossing rates induced by the heavy metal ions. The work shows that probing with metal ions is a useful method for studying accessibility of phosphorescent groups on proteins.

Significance to Biomedical Research and the Program of the Institute:

The spectrofluorometer was delivered in this institute some 20 years ago, and fluorescence spectroscopy largely grew as a result of that development. By continuing the activity of these laboratories in the area of fluorescence methods, we continue to advance a technique which has been of great utility in biomedical science.

Proposed Course:

The analysis of decay curves with the ORTEC is still not simple enough to be done by the average biochemist. We plan to see whether we can simulate 2 and 3-component decays with simple model systems or computer programs and to see what pitfalls one meets in trying to compare actual and theoretical multi-component decay. The time emission spectral system will be tested, and the effect of deuterium on the lamp output will be studied. In the phosphorescence studies, we would like to see if dried material at room temperature can be studied, as there are several reports that this can be done. Use of phosphorimetry has been severely limited by the need to operate at liquid $N_2$ temperature.

Key Descriptors: Fluorescence, tryptophane, silver ions, spectrofluorometer.

Honors and Awards: None

Publications:

1. Chen, R. F.: The Effect of Metal Cations on Intrinsic Protein Fluorescence, in Concepts in Biochemical Fluorescence, R. F. Chen and H. Edelhoch, Eds. M. Dekker, N.Y., in press.

Project No. Z01 HL 01409-04 LTD
1. Laboratory of Technical Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: An Automated Method for Rapid Bacterial and Mammalian Cell Growth and Assay

Previous Serial No.: NHLI-76

Principal Investigator: Peter Carmeci

Other Investigators: None

Cooperating Units: Medicine Branch, NCI
Dr. Joan Bull

Division of Oncology
Albany Medical College
Dr. Robert Sponzo

American Instrument Co.
Silver, Spring, Maryland

Project Description:

The objectives of this project are twofold; the first is to adapt the capillary scanning instrument to the various requirements of biomedical research by cooperating with potential users of the method to develop the techniques necessary to facilitate the utilization for clinical and research application. The second is to develop other methods where this technique is not applicable. At the present time efforts have been expended in the following areas of endeavor.

A. Developing methods of pulse height discrimination for
(1) segregating types of bacterial colonies during incubation, and
(2) studying the effects of growth factors and environment on mammalian cells.

B. Developing methods for early detection of mycoplasma (pneumonia).

C. Developing methods for assaying bone marrow stem cells.

a. A bread-board model pulse height discriminator has been built and the growth of stem, myeloma, hepatoma cells has been demonstrated. In addition, it has been found that a measurement of total growth of all viable colonies in a capillary, i.e., the integration of light scattered pulses, provides

another distinctive and sensitive parameter for growth and pulse height discrimination, have been designed and fabricated in a new unit that is presently undergoing test. Subsequent work will be to characterize the growth of myeloma, hepatoma, and stem cells under specific environmental conditions.

b. Previous indications have shown that mycoplasma can be detected by scattered light within 48 hours (compared to 10 day incubation period normally required). Growth in agar has not been conducive to growth. Growth in broth and in thin films of agar has been successful but not optimal. A new technique, based on the previous experience of growing, mycoplasma on thin films of agar, has shown more optimal growth. This consists of introducing the mycoplasma in solution to the surface of a thick (2mm) surface of agar in a number of thin lines that are optically aligned to the capillary scanner. Further testing of this technique is under way.

c. A new method of culturing human bone marrow stem cells has been developed. The cultures are grown in large plastic test tubes and the stem cells grow more profusely and reliably than in Petri dishes or capillary tubes. This was shown in a series of tests with human stem cells wherein samples were extracted into capillaries at various intervals during their growth in large tubes. This new method has caused us to investigate the possibility of detection assay within the larger test tube directly. The colonies are very few in relation to the total volume of the culture flask, therefore, to detect these by light transmission would be very difficult. Although the media scatters a lot of light, liquid scattering seems to be the easiest method for detection and assay.

When colonies are small, grown in a three dimensional solid media, and appear against a bright background of light scattered from colloidal media conventional colony counters fail. Computerized image analysis is elaborate and expensive. A simple approach has been developed for quantitating colony growth by using spatial frequency analysis to detect small colonies against a uniform bright background. Spatial frequencies are converted into temporal frequencies by chopping the image with a rotating optical pattern wheel, a method developed for use in missile guidance systems to detect small emitting targets against bright clouds. A prototype system, has been developed and applied to monitoring the growth of granulocyte colonies in methylcellulose medium with bone marrow suspensions, with good results.

Significance to Biomedical Research and the Program of the Institute:

The utilization of capillary tube scanning techniques and spatial frequency analysis with a rotating reticle in bacteriology and mammalian cell cultures. This will provide a) means of detection and assay early in the growth of colonies and b) methods that are readily adaptable to automation. The application to the study of cell metabolism, metabolic defects, oncology, and clinical cell sample assay is anticipated.

Proposed Course:

A. Develop hardware for pulse height analysis and investigate its ultilization for segregation of two or more organisms by their growth rates, and characterize cell growth under varying conditions.

B. Develop techniques to apply cappillary scanning for detecting and measuring mycoplasma (Pneumonia).

C. Optimize the technique for bone marrow stem cell assay and develop methods for sizing of cell colonies.

Keyword Descriptors:

Automated method, Mammalian cell growth, Cell colony monitoring, Capillary tubes, and Spatial frequency analysis.

Honors and Awards: None

Publications: None

Project No. Z01 HL 01410-01 LTD
1. Laboratory of Technical Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Blood Gas Monitoring for Extended Periods

Previous Serial No.: None

Principal Investigator: Gerald G. Vurek

Other Investigators: Theodor Kolobow and D. Warnock

Cooperating Units: Laboratory of Kidney and Electrolyte Metabolism, NHLI

Project Description:

Objectives: There are clinical situations and research problems in which frequent measurement of blood $PO_2$ and $PCO_2$ is desirable to follow the progress of therapy or result of experimental intervention. At present, the most reliable method is to withdraw blood samples and make the measurements with some specialized apparatus more or less remote from the source. The objective of this program is to explore a way to make an instrument perform these measurements at the point of use and yet introduce no more complicated apparatus than is presently used to monitor intravascular pressures.

Methods Employed: Reliable long term measurements require stable transduction systems, simple extraction techniques, and signal processing. Conventional polarographic oxygen sensors and Severinghaus $CO_2$ sensors require frequent calibration checks which make them unsuited for *in vivo* use. For oxygen, we use a galvanic cell which produces a current stoichiometrically related to the amount of oxygen supplied in the cell. A flow rate of $10^{-12}$ moles $sec.^{-1}$ yields a current of 0.3 μA which is easily measured. For carbon dioxide, we use our previously developed calorimetric measurement of the heat of reaction of $CO_2$ with LiOH. This also has picomole sensitivity. Of course, sample acquisition must be reliable because errors here cannot be corrected without reference to an alternative measurement. Our approach is to use a membrane covered probe which allows the gases in the blood to diffuse through the membrane in proportion to their partial pressures. By making the membrane permeability small compared to water (plasma) errors due to the ever present stagnant layer can be reduced. In addition, the probe can be made of materials which should resist changes due to imbibition of substances from the blood and by a process which should allow the properties of each probe to be measured prior to use. A simple but reliable technique for converting the signals to useful values is to convert the signals to digital form as close to the transducers as

possible to minimize the errors due to drift and nonlinear effects of analog circuitry. Thus, the blood gas system will use stable, sensitive, and specific transducers for gas analysis, a stable probe, and digital signal processing to obtain reliable long term blood gas monitoring.

Major Findings: Oxygen measurement with the galvanic cell is a well established procedure and is used industrially as well as in at least one electronic "Van Slyke" apparatus. We have found the commercial galvanic cell to be satisfactory although bulky. The sensitivity of the apparatus requires that all the components be gas tight to prevent atmospheric oxygen from leaking into the system and swamping the desired signal. At present we use Swagelok fittings which are satisfactory but leave room for improvement in terms of convenience.

Results from improvement on the apparatus for assay of picomole amounts of $CO_2$ (see FY 1974 report NHLI-87) now allow us to measure $CO_2$ with a sensitivity $\pm$ 1 s.d. of about $10^{-13}$ mole $sec.^{-1}$. This has been accomplished by using less noisy amplifiers and power supplies. Previous work (FY 1974 NHLI-127), had demonstrated that continuous measurement of $CO_2$ was difficult due to the problem of maintaining the proper water vapor content of the LiOH. This has been eliminated by using a sampling technique in which samples of the gas steam from the probe are periodically introduced into the calorimeter chamber which is a modification of the picomole assay apparatus. By using this sampling approach, we can eliminate the overshoot effect observed with the earlier steady state approach. In addition baseline drift of the calorimeter is corrected between samples. Samples can be taken at two minute intervals or less so that the overall system response time to changes in the patient's $PCO_2$ is adequate for monitoring purposes.

Sample probe design is dependent on several factors including relative permeability, response time, and convenience. In order to reduce the error due to the pressure of an unstirred layer of fluid adjacent to the probe, the overall permeability of the probe must be about 10% that of water. Oxygen is the limiting gas here for it is less soluble than $CO_2$ in most materials. Silicone rubber has excellent biocompatibility but it is 10 times as permeable as water so that a very thick, and thus very slow, membrane would be needed. Our present approach is to use a composite structure consisting of an open spring coated with a thin layer of silicone rubber, an intermediate coating of low permeability gas phase deposited para-xylylene, and an outer coating of silicone rubber. Para-xylylene has about $10^{-4}$ the permeability of silicone rubber so that a thin (1 um) layer has the needed permeability and rapid response. The silicone rubber layers are support and protection, and variations in their properties due to manufacture on imbibition produce second order effects on the overall probe response. At present we can use a 1 mm dia. by 15 mm long probe to obtain adequate gas flux at expected partial pressures of 80 mm Hg ($PO_2$) and 40 mm Hg ($PCO_2$).

Significance to Biomedical Research and the Program of the Institute:

Long term extracorporeal oxygenation and other forms of intensive respiratory care can be facilitated by knowledge of the status of blood gases. It is the purpose of this program to demonstrate an apparatus which can provide this information without the need for blood samples and with no more than a single catheter, comparable to a pressure manometer catheter, attached to the patient. Thus a step in the direction of biochemical monitoring will be made. This represents a quantum jump in intensive care monitoring because here-to-for only pressures and bioelectric signals have been monitored satisfactorily.

Proposed Course: The apparatus will be evaluated in vitro and its performance will be demonstrated in vivo. Additional development effort may be undertaken on the probe to make its piping more flexible.

Keyword Descriptors: Blood gas measurement, intensive care monitory, $PO_2$, $PCO_2$, galvanic cell, and microcalorimetry.

Honors and Awards: None

Publications: None

Project No. Z01 HL 01411-09 LTD
1. Laboratory of Technical Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Blood Flow Measurement Using Nuclear Magnetic Resonance Techniques

Previous Serial Number: NHLI-83

Principal Investigators: Vsevold Kudravcev, Robert L. Bowman

Other Investigators: Anthony Sances, Jr., Joseph H. Battocletti

Cooperating Units: Medical College of Wisconsin, Milwaukee, Wisconsin, on contract to Laboratory of Technical Development

Project Description:

To continue to develope the practical aspect of nuclear magnetic labelling technique for biological blood flow measurement, especially cerebral and digital (investigation of Raynaud's phenomena).

Progress: The work is divided between this laboratory and a group under contract to this laboratory at the Medical College of Wisconsin, directed by Dr. A. Sances, with Dr. J. H. Battocletti as a co-principal investigator. This group conducts the biological observations using the NMR labelling technique, performs theoretical analysis of the data obtained, and developes cerebral NMR flowmetric devices.

In this laboratory, the universal weak detector field blood flow meter previously constructed was investigated using simulated blood vessels (rubber tubing with an inside diameter of 1-2.5 mm). Satisfactory NMR results were obtained in the detector magnetic field with a strength of 20 gauss; a magnetic field of 2.5 kgs was used for liquid prepolarization. Signal-to-noise ratio was obtained in values of 20/1 and 10/1, depending upon flow velocity and simulated blood vessel volume, and without interference from surrounding tissue protons (simulated by the thick rubber tubing walls).

Helmholtz coils previously used for production of the NMR detector field were substituted by more effective and homogenous field coils. These newer coils were constructed to be large enough to accommodate human limbs and bodies of small animals, and will have practical application in the near future. This application is dependent on the development of proper polarizing field units of sufficient size and strength, and the completion of suitable NMR probes under construction at the present time.

A combined (single and cross coil) NMR probe was also constructed for digital blood flow measurement using a medium strength detector field (600 gauss). At this field strength, composite NMR signals appear, consisting of strong dominant NMR signals from polarized protons imbedded in surrounding tissues, and relatively weak signals from flowing blood. A data retrieval computer (10 averages) was necessary to separate this interferring, dominant signal from the true flow signal. Blood flow measurement in the human finger was successfully performed using a suitable computer during the author's recent visit to the Wisconsin facility. During this visit, NMR apparatus constructed individually by the two laboratories was tested and compared, and mutual exchange of equipment was undertaken with beneficial results to both research units.

During the past year, several of our basic NMR detectors were improved using recent developments in solid state technqiues and further modifications were implemented with resulting increase in stability and sensitivity. Improved envelope detector, low noise RF gates, dual gate FET preamplifiers, adjustable bandpass, L.C. filters, and many other innovations were incorporated in the apparatus circuits.

Major Findings: A new NMR flowing liquid magnetization envelope modulator was developed and constructed. The operation of this modulator is based on the superimposition of three separate magnetic fields: The DC magnetic field, the low frequency AC field, and the Larmor frequency RF field. This modulator allows periodically tagged magnetization envelope of the flowing liquid without switching the transient which is present in conventional NMR tagging gates. In addition, due to the fact that the DC field is supplied by the modulator himself the former may be moved over the blood vessels under investigation without continuous readjustment of the Larmor frequency required to produce NMR "burnout" tags, or needed degree of liquid magnetization envelope modulation.

Significance to Biomedical Research and the Program of the Institute: Non-intrusive NMR methods can be used to mesure peripheral blood flow or blood flow from various organs. This NMR system is therefore applicable for screening, continuous monitoring during surgery, or for the determination of blood flow in post-surgical or trauma patients.

Proposed Course: To apply the digital blood flow measurement technique developed previously for investigation of Raynaud's phenomena; cooperative research with the Medical College of Wisconsin for biomedical application; development of a practical version of the time-sharing and suppressed-carrier NMR SR detector described in previous reports.

Keyword Descriptors: Non-Invasive Blood Flow Monitoring, Nuclear Magnetic Resonance, NMR Magnetic Labelling Technique.

Honors and Awards: None

Publications:

1. Battocletti, J. H. Linehan, J.H., Wang, O.S., Sances, A., Larson, S. J., Evans, S. M., Itskovitz, H. D., Bowman, R. L., and Kudravcev, V.: Organ Blood Flow Measurement Using NMR. Digests Intermag. Conf., Toronto, Canada, 35.8, May 1974.

2. Battocletti, J. H., Sances, A., Larson, S. J., Evans, S. M., Bowman, R.L., Kudravcev, V., and Halbach, R. E.: A review of Nuclear Magnetic Resonance Techniques Applied to Biological Systems. In Llaurado, J. G., Sances, Jr., A., And Battocletti, J. H. (Eds): Biologic and Clinical Effects of Low-Frequency Magnetic and Electric Fields. Charles C. Thomas, Springfield, Illinois, 1974, pp 263-294.

3. Battocletti, J. H., Evans, S.M., Larson, S. J., Sances, A., Bowman, R. L., Linehan, J. H., Kudravcev, V., Genthe, W. K., Halbach, R. E., and Antonich, F. J. Measurement of Blood Flow by Nuclear Magnetic Resonance Techniques. In W.E. Vannoh and H. Wayland, (Eds): Flow, Its Measurement and Control in Science and Industry. Instrument Society of America, Pittsburgh, Pa., 1974, Vol. 1, Part 3, pp 1401-1409.

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5. Battocletti, J. H. Sances, Jr., A., Larson, S. J., Evans, S. M., Bowman, R. L., Kudravcev, V., and Ackmann, J. J.: Clinical Applications and Theoretical Analysis of NMR Blood Flowmeter. Biomed. Eng. (London) 10 (1): 12-20, January, 1975.

Project No. Z01 HL 01412-03 LTD
1. Laboratory of Technical Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Discrete Cell Temperature Measurement Study

Previous Serial Number: NHLI-113

Principal Investigator: Robert L. Bowman

Other Investigators: E. Ronald Atkinson

Cooperative Units: J. Peterson, Biomedical Engineering and Instrumentation Branch, DRS.

Project Description:

Objectives: Radioautography and staining methods are the means generally available for indication of cell response to mitogens, immunoreagents and specific metabolic stimulants, i.e. lymphocyte transformation tests. We are examining, developing instrumentation and methods to grade cell responses to these metabolic modifiers by measuring heat output from discrete cells. We hope to provide a visual indication of heat production from each cell in a field observed microscopically.

Methods:

1. A system based on a curve point transition in a thin slice of ferroelectric material which changed its optical properties at 35°C was abandoned when the reputed transition point was observed to be too high for use and no other suitable material was available.

2. A system based on the rate of condensation of a volatile oil (dimetyl silicone) on a thin film that supports a film of cells under a cover glass uses the heat evolved from each cell to modify the thickness of the condensation film which is observed as interference fringes appearing around each cell. A thermal (Peletier) effect heat pump below the film is cycled to condense and volatilize the oil below the film.

3. Cholesteric liquid crystals with a metastable state in 35-37°C range are formed in films to produce a color background that will be modified by local cell heat.

4. Radiometric image formation by use of a superconductive bolometer based on the point of superconductive transition of niobium. Direct radiation from single cells could be measured.

Major Findings: The evaporating film system has been constructed and tested with several systems last year and discrete cells were observed to modify the oil film thickness to produce patterns indicating that some cells were better heat sources than others but controls that would preclude the possibility that the fringes were artifacts were needed. This year fresh granulocytes were mixed with bacteria (E. Coli) and the evidence of heat compared to number of bacteria ingested. These experiments showed a high heat production related to number of organisms ingested but on occasion controls without bacteria also showed heat production which was presumed due to degranulation and lysis of older cells.

A modification of the system of introducing cells that will permit addition and removal of reagents to the cells while they are observed will be used to establish internal control and avoid artifacts.

The liquid crystal approach using available cholesteric substances had a texture of color and crystalinity due to local color domains of the same order of magnitude as the cells. A program to purify the liquid crystal material at the Chemical Engineering Section of BEIB has demonstrated remarkable improvement in the uniformity of films and suggests that a $37^{o}$ material should be processed and tested on cell suspensions.

No suitable crystal with the requisite transparency and ferroelectric curie transition at physiological temperatures and suitable mechanical properties is available and this approach has been suspended until such material becomes available. The superconductive bolometer approach has been carried to the stage of demonstration that the niobium bolometer performs with the sharp change that was anticipated but this approach is expected to lead to a relatively cumbersome and expensive system that needs more investment in time, personnel and money than is wise to invest until the simpler systems are proven inadequate or specific requirements identified that can only be met by the radiometric system.

Significance to Biomedical Research and the Program of the Institute: Single cell measurements may be particularly important when specific reactor cells cannot be separated from bulk cells. A few reacting cells in a larger batch may be particularly important in oncogenesis, aging, and immuno-reactions.

Proposed Course: Refinement of sample handling, determination of sensitivity, improvement of microsocpic image and documentation of results of specific assays.

Key Descriptors: Microcalorimetry, Lymphocyte Transformation, Phagocytosis, Cell Metabolism, and Radioautography.

Honors and Awards: None

Publications: None

Project No. Z01 HL 01413-13 LTD
1. Laboratory of Technical Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Instrumentation for the Study of Pre-Steady State Enzyme Kinetics

Previous Serial No.: NHLI-74

Principal Investigator: Robert L. Berger

Other Investigators: J. Frolich, NICHD
M. Marini, Dept. Biochem., Northwestern Medical School
N. O. Kaplan and J. Everse, U. of California, at San Diego Medical School
L. Rossi-Bernard, University of Milan, Italy
J. A. McCray and P. Smith, Dept. of Physics, Drexel U.
W. F. Friauf, H. Cascio and E. Beile, BEIB
R. Shrager, DCRT, Lab. Physical Sciences
M. Sapoff, Thermometrics, Inc.
B. Balko, Dept. of Physics, Boston University

Cooperating Units: Ingold Electrodes, Zurich

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 solutions and in the intact cell or cell membrane. In particular, to develop the method and instruments to study, in collaboration with other laboratories, the reactions of hemoglobin with the respiratory gases both in the normal state and as modified by the changes of physical factors, small molecules, various metabolites, and gentically, such as in sickle cell anemias. The reactions of various cellular enzymes, particularly ATPase and lactate dehydrogenase, and their interactions, and control, in the cell are studied as they relate to the hemoglobin reactions in cardiology, pulmonary and respiratory function, and 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 reactions by optical, thermal, glass electrode, etc., detectors. In general, equipment is not available, either

in the literature or commercially, for investigations in this area. Such apparatus is conceived and designed in this laboratory, together with consultants, construction being carried out wherever most appropriate; i.e., in our shops or by commerical firms, special university facilities, or at the several special research laboratories such as the Jet Propulsion Laboratory. In pursuing these investigations, a wide variety of physical parameters must be studied, which leads to the need for an understanding of the underlying physical theory governing the reactions. Expert consultants and collaborators are brought in to assist in the design, analysis, and evaluation of the equipment, particularly as it applies to certain specific enzyme systems under investigation.

Major Findings:

The optical stopped-flow system has been further improved by the development of a new type of observation tube which has eliminated several artifacts found when the system was put under stringent tests with a biochemical system (EGTA & Ca). Improvements in the high speed stop valve have greatly increased the reliability of the instrument and again eliminated a very troublesome artifact.

A rapid chemical quench mixing apparatus has been developed for the study of fast enzymatic reactions containing chemically stable reaction products. The essential parts of the apparatus are: (1) syringe block and mixers, (2) stepping motor and drive assembly, and (3) assay value. Solutions containing enzyme and substrate are driven through a mixing chamber (Berger ball mixer) and allowed to react in an intermediary tube before passing into a second mixer where a quenching agent is added to terminate the reaction. Alternately, substrate additions can be made in the first and second mixers and the quenching reagent added to a third mixer. Reaction time is varied by changing the volume of the intermediary tube and the flow velocity of reactants between mixers. The syringe pistons are actuated by a common platform attached to a lead screw which converts the rotational motion of the stepping motor to linear translational motion. Fluid emerging from the final mixer passes through an assay valve which shunts material remaining in the line from the previous shot to waste before a new sample is collected. The apparatus, which was calibrated by measuring the pseudo-first order rate constant for the alkaline hydrolysis of 2,4-dinitrophenylacetate, has a dead time (minimum quenching time) of .003 seconds.

The instrument is currently being used to study the pre-steady state time course of ATP hydrolysis by the ($Na^+$-$K^+$) activated ATPase of electroplax microsomes. This activity is part of an enzyme system which couples active $Na^+$ extrusion to inward $K^+$ flux across the plasma membrane. In the presence of $Na^+$ the microsomes are rapidly phosphorylated by ATP resulting in an acid-stable intermediate complex, E ~ P. If $K^+$ is added with $Na^+$ phosphoprotein degraded to an acid-labile intermediate resulting in an

overshoot in the E ~ P vs time curve and an initial "burst" of inorganic phosphate production. Breakdown of the acid-labile intermediate designed E·P in the following sequence is rate limiting at low ATP:

$$E + S \underset{(1)}{\longrightarrow} E.S. \xrightarrow[(2)]{Na^+} E \sim P \xrightarrow[(3)]{K^+} E\cdot P \xrightarrow[(4)]{ATP} E + P_i$$

At high ATP substrate activation of the final step is observed. Although the precise relation of steps in the enzymatic and transport processes are yet unknown the fact that E ~ P is activated by low concentrations of $Na^+$ suggests that it represents a high affinity state of the enzymatic carrier for $Na^+$. By analogy E·P which is formed at low concentrations of $K^+$ may show high affinity for $K^+$.

A new differential thermistor bridge has been constructed and tested with excellent results. It will be used on both the high speed stopped flow calorimeter, recently completed, with a new thermistor probe which now has excellent stability and very low leakage. Extensive testing will commence as soon as all components are operating reliably. See the attached appendix for the complete mathematical simulation of this system and the detailed design of the calorimeter. This is the report of Dr. Balko who contributed to the detailed design and testing of this system. Preliminary experiments on glutamic dehydrogenase reactions have been carried out with Dr. Harvey Fisher, V. A. Kansas City, Kansas, furnishing us material and biochemical guidance.

A new differential high speed-high sensitivity pH meter has been finished and tested at Ingold Electrodes on a fluoride detection system where one electrode was a potassium electrode and the other a fluoride electrode which is also sensitive to potassium. Results show that the electrode can be used to $\pm$ 0.0001 pF. It will be coupled with the differential thermistor system to do thermal-potentiometric protein titrations simultaneously.

Much work on the coating of pH electrodes has been carried out using Lycra. Excellent results with one set of electrodes were obtained but so far the work does not seem to be repeatable on other than a specific type of glass electrode.

Significance to Biomedical Research and The Program of the Institute:

An understanding of the basic mechanisms of disease is a prerequisite to prevention and cure. The investigation of the reaction of the respiratory gases with hemoglobin, the red cell, and cytochrome oxidase in heart muscle cells is fundamental to an understanding of normal cell respiration and particularly to what has gone wrong as in the case of sickle cell anemia, mycardial infarction, etc. It is hoped that this research will result in instrumentation to permit the medical scientist to perform research leading to clarification of the ways in which, for example, sickle cell anemia can

be managed by the use of chemicals. The extension of such investigations to other disease systems and possible results seem abundantly clear in terms of preventive medicine and improvement in health care.

Prposed Course: Work will continue on the various systems and detectors to bring each instrument to the point where its usefulness to the biomedical scientist has been established. Efforts will then be made to have it available from manufacturer.

Keyword Descriptors: Fast Thermistors, Thermistor Bridge, Stopped-Flow Calorimeter, Quenched-Flow Apparatus and ATPase Sacroplasmic Reticulum.

Honors and Awards: None

Publications:

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Project No. Z01 HL 01414-03 LTD
1. Laboratory of Technical Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Development of Microcalorimeters for Clinical Chemistry

Previous Serial No.: NHLI-75

Principal Investigator: Robert L. Berger

Other Investigators: Edwige Panek, Frank Noble, Technical Development, NHLI
Donald Young, Nadja Rehak, Clinical Chemistry, NIH
Edward Prosen, Physical Chem. Div., NBS
Luigi Rossi-Bernardi, Prof. Enzymology, U. of Milan
Mario Marini, Assoc. Prof. Biochem., Northwestern Univ.
Norman Davids, Prof. Eng. Mech., Penn State Univ.
Bohdan Balko, Dept. of Physics, Boston Univ.

Cooperating Units: BEIB, LMH, NHLI

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 equilibration 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 a completed instrument 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: The batch calorimeter has been used both in this laboratory and in clinical chemistry to investigate its use and limitations on several specific reactions.

The calorimetric system for measuring the heat of reactions was set up in the normal mode (it's measurement of total heat of reaction) using an integrator built in BEIB and calibrated with acid-base reaction.

Determination of uric acid in serum was initiated and compared with the method used in the clinical laboratory. Effect of protein level in the serum on the calorimetric determination of the uric acid is being evaluated.

Preliminary investigation of the hydrolysis of hemoglobin by pepsin and trypsin in model reaction and gastric juices established the feasibility of the calorimetric method in the kinetic mode for determination of the activity of these enzymes in gastric juices and feces.

The interfacing of the microcalorimeter with the computer system is now finalized and the system is ready to be used in the kinetic mode. Using synthetic substrates which are specific for either pepsin or trypsin only, the calorimetric determinations of these enzymes is compared with the routine spectrophotometric method.

Determination of cholesterol in human serum using the cholesterol oxidase-catalase coupled reaction was carried out on the batch-type, NBS-NIH microcalorimeter at 37°C. The experiments were performed either in phosphate or tris-HCl buffers, at pH 7.4. Because of the low specific activity of the cholesterol-oxidase (ranging from 0.4 IU to 5 IU of the enzyme commercially available), and its poor affinity for the substrate, the amount of enzyme has to be high enough to transform the cholesterol to cholest.4 en-3 one., therefore, the two compartments of the cell have to be balanced with an equal concentration of albumin in order to avoid any heat of dilution. Under those conditions, the cholesterol-oxidase and catalase coupled reaction is probably partially inhibited by the low concentration of oxygen available. To improve this methodology the cholesterol oxidase (5 IU/mg) is being attached to glass beads.

The determination of triglycerides was carried out by the same method as described for cholesterol. The first step of the analysis involves the enzymatic hydrolysis of the triglycerides by lipase in a phosphate buffer at pH 7.0. The $\Delta H$ for that reaction was demonstrated by micro-calorimetry to be close to zero. The same result was detected from the heat of combustion of pure tiplomitin. In the second step, the glycerol liberated from the enzymatic hydrolysis was coupled to glycerokinase and ATP, the $\Delta H$ of the reaction was 6.5 k cal/mole, corresponding to the ATP hydrolysis. However, it was observed that an endothermic reaction preceeded the ATP hydrolysis exothermic reaction. This corresponds to a contamination of the glycerokinase by a low ATPase activity. This undesirable secondary reaction, limits the sensitivity of the method to 0.1 M of glycerol or triglyceride.

The stopped-flow microcalorimeter is undergoing extensive testing to eliminate a number of artifacts that make its operation less reliable than the batch system at present. The mathematical simulation used in design and data correction of these systems in available as an in house technical Report.

Significance to Biomedical Research and the Program of the Institute:

The use of these methods as an analytical tool for clinical chemistry shows

considerable promise as a means of improving the accuracy, precision, and thruput of clinical tests. In addition, it makes possible the use of many new tests for enzyme or substrate tests, antigen-antibody reactions, coagulation tests, etc., which are not now able to be done due either to the lack of a suitable detection method or to the fact that the present tests are long, have high variability, and are therefore not used.

Perhaps the long-range significance of this project are the possibilities that the calorimeter offers for the study of many biochemical reactions which cannot now be investigated due to a lack of a suitable detector of the reaction. An example of current interest in the many steps preceeding final coagulation that occurs in the forming of a thrombus.

Proposed Future Research: The effectiveness of the batch microcalorimeter as an instrument suitable for routine clinical work will continue to be explored in collaboration with clinical chemistry and molecular hematology. Additional exploration of other chemical reactions is planned particularly in the area of fatty acid binding to proteins and antigen-antibody reactions. Considerable work is needed to solve a number of technical problems associated with high reliability and sensitivity of the stopped-flow microcalorimeter and these will continue to be pursued. The titration calorimeter needs additional work particularly in regard to the inclusion of pH electrodes for simultaneous pH-thermal titrations of proteins and this will be vigorously pursued in the next year.

Keyword Descriptors: Stopped-Flow Microcalorimetery, Cholesterol Cholesterol Oxidase, Triglycerides, glycerokinase, 2-3 DPG, Batch Microcalorimeter.

Honors and Awards: None

Publications:

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2. Berger, R. L., Friauf, W. S., and Cascio, H. E.: A Low-Noise Thermistor Bridge for Use in Calorimetry, Vol. 20, pp 1009-1012, 1974.

3. Goldberg, R. N., Prosen, E. J., Staples, B. R., Boyd, R. N., Armstrong, G. T., Berger, R. L., and Young, D. S.: Measurements Applied to Biochemical Analysis: Glucose in Human Serum. Anal. Biochem. in press.

Project No. Z01 HL 01415-02 LTD
1. Laboratory of Technical Development
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Italy-U.S. Cooperative Science Program - Blood Gas Instruments - Project 78.

Principal Investigators: Robert L. Berger
Luigi Rossi-Bernardi, University of Milan, Milan, Italy

Other Investigators: M. Luzzana, University of Milan
R. Winslow, Lab. Molecular Hematology, NHLI

Cooperating Units: Ingold Electrodes, Zurich
Advanced Products, Milan, Italy

Project Description:

Objectives: The total oxygen needed by a normal healthy subject is provided by the circulatory system according to the well known equation:

$O_2$ consumption = cardiac output x arterial-venous $O_2$ difference.

Since several pathological conditions can shift the oxygen dissociation curve (ODC) and thus how much oxygen can be released to the tissues, it is of considerable clinical and fundamental physio-chemical 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 various physiological or pathological conditions. ODC position and shape is under control of various small molecules or ions, i.e. $CO_2$, protons, and 2,3-DPG, etc.

Methods Employed: A systematic analysis of the complex interrelationship among such variables and their effect on ODC requires the development of a simple method to obtain ODC of human blood, in vitro, under conditions closely simulating the in vivo situation of the patient.

Instruments are developed either at NIH and/or Milan, tests on pure hemoglobin are generally conducted first in Milan where a large group is currently working on the purification of hemoglobin. Testing on patient blood is then carried out in Molecular Hematology. Close cooperation exists with the medical school hospital in Milan where on-line work will be

carried out using the membrane oxygenator system, developed in this laboratory by Dr. Kolobow, monitored for % $O_2$ Hb by a modification of the Optisat also developed here.

Major Findings: A semi-micro (ODC) apparatus has been constructed and tested both in Milan and here. It consists of a tonometer for degassing the blood (.5 to 2 ml's needed), a cell containing .5 or 1 ml of the degassed blood, an oxygen electrode, a $CO_2$ electrode, two syringe drives for adding $H_2O_2$ and NaOH continuously, and an x-y recorder. About 20 to 25 minutes is needed for degassing the blood. .5 ml is transferred to the ODC apparatus which contains 10 µl of catalase. Stirring is started, zero $O_2$ determined and $CO_2$ noted. $H_2O_2$ is then added continuously and this addition plotted as the abscissa. The $O_2$ electrode reads $O_2$ in solution and thus is proportional to the percent oxyhemoglobin. NaOH is added to keep $CO_2$ constant. Then the ODC curve is run under near physiological conditions in about 10 minutes. The system was carefully checked against the Van Slyke manometric apparatus. A number of corrections for dissolved oxygen, methemoglobin formation, dilution from addition, etc. have been worked out. The system has been completely automated on a PDP-8 computer so that the $H_2O_2$ dirve and NaOH addition are controlled, calculations made, and both printout and plots carried out. In the accompanying graph one sees that in a curve for sickle cell blood the P50=41 mm of Hg, while for normal HbA blood it is 28 mm of Hg. Of greater importance is the fact that the frozen and thawed hemolysate of the sickle cells falls on the same curve as normal RBC's. From a clinical standpoint, only a system which measures whole blood, keeping $CO_2$ constant during the run, gives an adequate picture of the systems ability to deliver oxygen. Thus, P50 or ODC measured on an instrument such as theco-oximeter can give erroneous results. Note particularly B of the figure which shows the total oxygen delivered to the system. Thus, it is crucial that a consideration of what shifts occur in the ODC and how that affects total oxygen capacity deliverable at normal venous partial pressures.

Significance to Biomedical Research and the Program of the Institute.

The development of a simple instrument for rapid determination of the oxygen dissociation curve on ½ ml of blood would be of great importance in estimating the status of infants during respiratory failure, operating room status of patients under anesthesia, and what is happening to the blood of patients with normal or abnormal hemoglobin during various forms of therapy, i.e. treatment of sickle cell patients. In addition, it allows us for the first time to conveniently do the many physiochemical determinations necessary to test models of hemoglobin action.

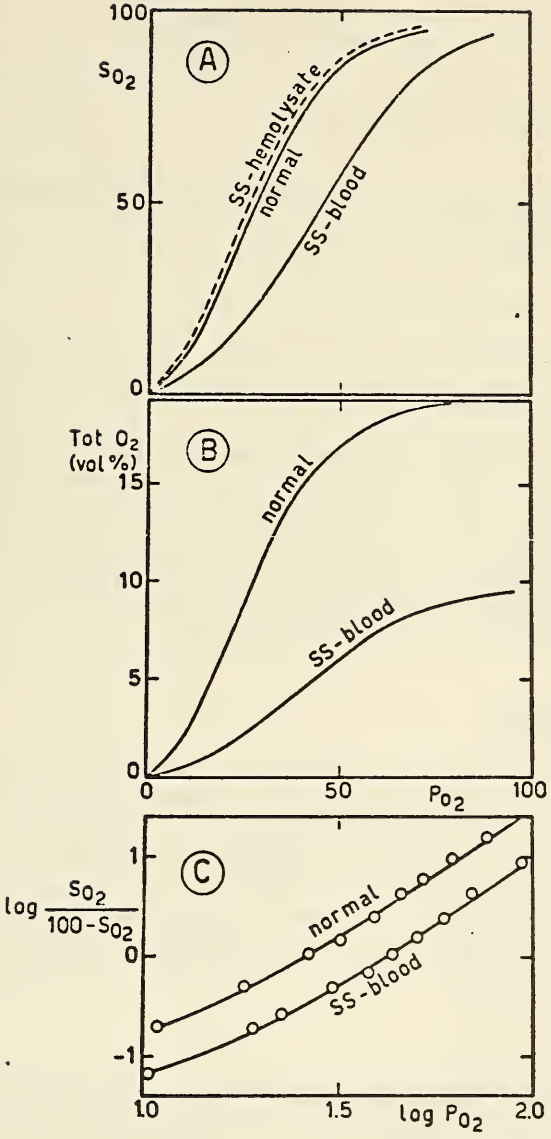
Proposed Course: A prototype of a new instrument to determine Hb, Met, $C_OHb$, $O_2Hb$ on 10 µl of blood is undergoing laboratory tests and will be put on clinical trials starting 1 July. A new microprocessor controller to run the ODC apparatus, make calculations, and plot, will be tested and added to the system thus producing a complete semi-micro blood-gas apparatus for determining pH, $PCO_2$, $PO_2$, ODC, and Hb.

Keyword Descriptors: Hemoglobin, Red Cell, and ODC Analyze

Honors and Awards: None

Publications:

1. Rossi-Bernardi, L., Rossi, F., Luzzana, M., Perrella, M., and Berger,R.L.: Physiological Properties of Sickle Cell Hemoglobin, in Proc. of the 1st Nat. Symp. on Sickle Cell Disease, DHEW Publication No. (N.I.H.) 75-723, U. S. Gov. Printing Office, 1974.


A
$S_{O_2}$
100
50
0
SS-hemolysate
normal
SS-blood
B
Tot $O_2$ (vol %)
15
10
5
0
normal
SS-blood
0
50
100
$P_{O_2}$
C
$\log \frac{S_{O_2}}{100-S_{O_2}}$
1
0
-1
normal
SS-blood
1.0
1.5
2.0
log $P_{O_2}$

Project No Z01 HL 01416-01 LTD
1. Laboratory of Technical Development
2. Section on Pulmonary & Cardiac Assist Devices
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: On-line cardiac Output Measurement During Extracorporeal Membrane Oxygenation

Previous Serial Number: None

Principal Investigator: Edward Stool

Other Investigators: Theodor Kolobow, Gerald Vurek, and Joseph Pierce

Cooperating Units: NHLI Surgery

Objectives: During extracorporeal membrane oxygenation (ECMO) cardiac output must be measured by somewhat cumbersome intermittent determinations (Fick, Indicator-dilution, dye or thermal. These procedures generally require additional personnel and therefore are relatively infrequently performed. Their intermittence makes them subject to sampling error in a situation where wide fluctuations in patient status are often seen. The objective of this study is to develop a technique for continuous cardiac output measurement of patients on extracorporeal bypass.

Methods:

(a) Theoretical Analysis

Utilizing the fact that the membrane oxygenator is continuously transferring oxygen into the patient's blood an attempt to use oxygen as a marker in veno-veno or pre-pulmonary bypass was undertaken. This can be done by either of two methods. The first method relies on determination of oxygen saturation in a continuous fashion across the membrane oxygenator and in the pulmonary artery and is complicated by the fact that it depends on the assumption that venous inflow to the membrane lung closely approximates "mixed venous" blood in its oxygen content. A second method eliminates this consideration but requires stopping pump flow for 5-10 sec. and hence is not in an absolute sense continuous.

(b) Experimental

Large closed-chest anesthetized sheep are peripherally cannulated in a manner similar to patients undergoing ECMO, however, a large bore return catheter is positioned in their right ventricle. High flow veno venous

ECMO is carried out while the appropriate oxygen saturation determinations are made. This is done under basal conditions, oxygen deprivation, β-adrenergic blockade and β adrenergic stimulation. Simultaneous cardiac output determinations by conventional technique are carried out and compared to those obtained by the oxygen indicator method.

Major Findings:

Preliminary experiments have been directed towards assessing optimal cannulation techniques and in determining instrument stability under operational conditions. Further, reproducible pharmacologic interventions to safely alter an anesthetized sheep's cardiac output have been developed.

Significance of the Program to the Institute:

ECMO is a therapeutic modality currently undergoing extensive clinical evaluation in a NHLI contract. The ability to measure cardiac output continuously during ECMO will not only improve the management of these patients but will serve as a powerful research tool to allow for better analysis of the physiologic effects of such interventions.

Proposed Course:

Current experiments will determine the precision of the two methods of cardiac output determinations as well as their degree of correlation with conventional cardiac output techniques. Following a suitable number of in vivo perfusions, instrumentation to render the proposed determinations truly "on-line" will be constructed.

Keyword Descriptors: Cardiac Output, Membrane Oxygenator, Extracorporeal Circulation.

Honors and Awards: None

Publications: None

ANNUAL REPORT OF THE
CARDIOLOGY BRANCH
NATIONAL HEART AND LUNG INSTITUTE
July 1, 1974 through June 30, 1975

The experimental interests of the Cardiology Branch developed over the past few years have continued. These relate to the pathogenesis, pathophysiology and treatment of coronary artery disease; the ultrastructural and molecular mechanisms responsible for normal and impaired contractile function of the heart; development of the diagnostic and investigational capabilities of echocardiography; and the application of multidisciplinary techniques to define the determinants of irreversible heart failure in patients with valvular disease and how such information can be used to determine optimal time for surgical intervention.

## CORONARY ARTERY DISEASE

### Pharmacologic Treatment of Acute Myocardial Infarction

In the past few years, we have shown that treatment with TNG following coronary artery occlusion in dogs diminishes infarct size and reduces the incidence of ventricular fibrillation (VF) occurring spontaneously during AMI. These actions are potentiated when a vasoconstrictor is administered to abolish the TNG-induced fall in arterial pressure and reflex tachycardia.

To elucidate the mode of action of TNG in AMI, we measured the effects of treatment on myocardial blood flow and on ischemic injury during coronary occlusion in dogs. We found that the salutary effects of TNG on ischemic injury during AMI are mediated by an increase in collateral flow and reduction in $MVO_2$. However, if TNG causes hypotension or excessive tachycardia, reduction of ischemic injury will occur only when a vasoconstrictor is administered to reverse the pressure and heart rate changes.

We are now evaluating this form of therapy on the extent and severity of myocardial injury sustained during AMI in man. Thus far we have found that in pts without left ventricular failure, TNG alone resulted in no consistent decrease in ischemic injury. However, when phenylephrine was added to reverse the blood pressure lowering and heart rate speeding effects of TNG, significant improvement in myocardial ischemia occurred uniformly. In contrast to the pts without failure, TNG alone significantly improved ischemic injury in all pts with failure.

### NHLI Type II Coronary Intervention Study

The primary aim of this randomized, double blinded, prospective study, carried out in collaboration with the Molecular Disease Branch, Section of Lipoproteins, is 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. 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 two years of treatment. The program is now well underway; some of the information that has emerged to date is detailed in another section of the Annual Report.

In addition to the primary question, the screening of numerous patients for entry into the investigation has led to several fall-out studies. For example, the ECG response to exercise has heretofore been used as a reliable test to screen for the presence or absence of coronary artery disease. In our just completed analysis of patients who had exercise studies and coronary arteriograms, we found that the ECG response to exercise yielded false-negative and false-positive results in over half of the subjects tested. This low sensitivity and specificity indicates use of this test as a diagnostic tool in the individual patient is questionable.

Prospective Study on the Natural History of Patients with Coronary Artery Disease with Only Mild to Moderate Functional Disability

Considerable information, obtained retrospectively, is available relating to the natural history of coronary artery disease. These studies suggest that mortality rate can be predicted by the number of diseased coronary vessels and the presence and magnitude of ventricular dysfunction. Using such data, "prophylactic" coronary bypass operation is being recommended if a patient, even if only mildly symptomatic, falls into a particularly high risk group. However, these studies are based on data obtained largely from severely symptomatic patients and may not accurately reflect long-term prognosis of the patient with minimal symptoms. Therefore, patients with only mild to moderate functional disability are being studied by cardiac catheterization, exercise testing, 24-hour ECG tape monitoring, etc. Attempts will be made to determine prognostic indices. If high and low risk subgroups can be identified, then more rational decisions can be made regarding which patient should be considered a candidate for "prophylactic" operation.

## AUTONOMIC INNERVATION OF THE HEART

Effects of Cardiac Failure on Ventricular Electrical Stability and Autonomic Innervation of the Heart

The autonomic nervous system has profound influences on the electrical stability of ventricular myocardium. Moreover, the parasympathetic and sympathetic systems have opposite effects. Increased vagal tone decreases the propensity of the heart to develop VF, while enhanced neural sympathetic tone increases the likelihood of developing VF. Since heart failure decreases cardiac neuronal stores of norepinephrine, we have studied the effects of failure on the electrical stability and autonomic innervation of the heart. We found that failure-induced depression of cardiac norepinephrine stores increases ventricular electrical stability. Moreover, cholinergic innervation of the ventricular septum was reduced or absent in most of the hearts derived from failure animals, a finding that correlated with impaired capacity of vagal stimulation to enhance VF threshold. Thus, cardiac failure reduces or eliminates autonomic neural influences on the heart. The relative magnitude of

the adrenergic and cholinergic impairment may, in part, determine the likelihood of heart failure leading to arrhythmic death.

## ECHOCARDIOGRAPHIC STUDIES

### Asymmetric Septal Hypertrophy, or ASH

By employing echocardiographic techniques, in the past two years we have considerably increased our understanding of the disease spectrum embracing IHSS. Of note, it was recognized that LV outflow obstruction was only one manifestation of a disease that is basically a cardiomyopathy characterized by a septum that is disproportionately thickened in relation to the posterior left ventricular wall. We also showed the disease is a genetic abnormality transmitted as an autosomal dominant trait with a high degree of penetrance.

This past year, we studied the clinical characteristics and course of 35 children with ASH followed for one to 16 years (average, 7.4 years). Although 52% of the 35 patients improved or remained stable, 17% deteriorated clinically and 31% died suddenly (4% mortality per year). Two of the patients who died suddenly had previously undergone operation (6 and 13 years previously) with resultant abolition of the outflow gradient; 4 others were taking propranolol. No indices predictive of sudden death could be identified. Thus, the clinical and hemodynamic spectrum of ASH in children is broad, and, unfortunately, sudden death is relatively common in that subgroup of children who were referred to the NHLI in the past because of overt manifestations of cardiac disease.

### Echocardiographic Assessment of Cardiomyopathies

We are continuing our studies of cardiomyopathy by echocardiography begun last year. We have accumulated considerably more patients and have confirmed our initial impression that an extremely useful clinical classification of the cardiomyopathies can be achieved by echocardiography. Patients have been divided into those with dilated cardiomyopathy, those with normal LV volumes with concentrically hypertrophied walls, and those with normal LV volumes with ASH. The secondary cardiomyopathies (alcoholic, amyloidosis, hypereosinophilia, hemochromatosis, mucopolysaccharidoses, etc.) fall into one of the first two groups; the third is a specific genetically determined disease. This classification system markedly simplifies diagnosis of patients presenting with a cardiomyopathy.

### Pathophysiology and Prediction of Onset of Atrial Fibrillation

Systemic embolization, a serious complication of mitral valve disease and of ASH, usually occurs in patients in atrial fibrillation (AF) and particularly in those who recently have converted from NSR to AF. In an attempt to more completely understand the pathophysiology of AF, echocardiography was employed to study 85 patients with isolated mitral valve disease, 50 patients with isolated aortic valve disease, and 130 patients with ASH. In all three groups of patients, AF was common only in the subgroup of patients older than 40 years of age who in addition had a left atrial dimension measured by echo that

exceeded 45 mm. Our data indicate that a chronic hemodynamic burden initially produces left atrial enlargement which in turn predisposes to AF. Of note, 12% of patients who had AF had an embolus at its onset. Thus, "prophylactic" anticoagulation may be indicated in a patient in NSR with mitral valve disease or ASH who has a left atrial dimension exceeding 40 mm.

Determinants of Ventricular Septal Motion

Normally, the ventricular septum moves posteriorly during systole. Certain conditions, however, lead to anterior or "paradoxical" movement. To define the determinants of septal motion, echocardiographic studies were performed in patients with a variety of cardiac disorders. We found that the direction and magnitude of septal motion is determined by septal position at end-diastole relative to total cardiac transverse dimension. The more posterior the septum lies (as with right ventricular dilatation) the more likely it will move paradoxically. Thus, although paradoxic septal motion is usually seen in conditions causing right ventricular volume overload, it is not diagnostic of any particular hemodynamic burden. Additional studies of cardiac motion employing two-dimensional echocardiography are compatible with the hypothesis that all intraventricular structures move during systole towards the center of ventricular mass. This hypothesis has broad implications in predicting cardiac motion in the normal and diseased heart, since it provides the theoretical basis governing altered patterns of cardiac motion.

Real-Time Two-Dimensional Echocardiography

Over the past two years, we have developed a sector-scanner that produces real-time, two-dimensional echocardiograms that permits visualization non-invasively of internal cardiac structures. We have found this technique to be a powerful tool for diagnosing and understanding the anatomic relations of many complex congenital anomalies. We also have found that this technique allows us to measure mtiral valve area in patients with rheumatic heart disease, even in the presence of mitral regurgitation. Heretofore, accurate assessment of mitral valve area could only be made by cardiac catheterization, and only if mitral regurgitation were not present.

SUDDEN INFANT DEATH SYNDROME

Sudden infant death syndrome (SIDS) is a major cause of mortality in the first six months of life, but the primary mechanisms responsible for this condition are unknown. To investigate possible cardiac mechanisms, 42 sets of parents (who had at least one infant die of SIDS) were studied by echocardiography and ECG. ASH was present in two (5%) parental sets. At least one member of 13 (31%) other parental sets had ECG abnormalities, the most common of which was QT interval prolongation. In addition, 47% of the living children of the parental sets with QT interval prolongation had the same abnormality, consistent with an autosomal dominant pattern of inheritance. We also studied three infants with "near-miss" SIDS. All three showed prolonged QT intervals. Thus, our data suggest that cardiac mechanisms, especially QT interval prolongation, may play a role in a considerable proportion of infant deaths falling within the sudden infant death syndrome.

## VALVULAR HEART DISEASE

### Elucidation of the Determinants of Irreversible Myocardial Failure

Last year we completed a retrospective study of long-term survival in patients operated on for aortic regurgitation. We found that although absolute heart size preoperatively did not influence long-term postoperative survival, change in heart size as assessed over the first 4-6 months following operation did. Thus, 85% of patients operated upon for aortic regurgitation whose cardiothoracic ratios decreased survived six years. In contrast, only 43% of patients ($p<.02$) whose heart size did not change or whose heart size increased survived six years. This prompted a prospective multidisciplinary study to define 1)whether a particular grouping of preoperative functional derangements leads to prohibitive operative risks, and 2) what type of derangements can be reversed or improved by operative abolition of the mechanical defect. Evaluation of myocardial function includes calculation of ventricular volumes, ejection fraction (EF), exercise testing, etc. In addition, biopsies are being obtained for electron microscopic analysis as well as biochemical assessment of contractile proteins. Preliminary analysis of the pre- and postoperative data of one group of patients - those with isolated aortic regurgitation, has been performed. The 20 patients studied pre- and postoperatively were divided into three groups based on preoperative EF: normal EF (>60%), intermediate EF (40-60%), and low EF (<40%). We found that 1) operation does not improve basal ventricular function, 2) LV volume and mass are more likely to return toward normal in patients with normal or intermediate EF, 3) long-term results are good in patients with normal or intermediate EF, and 4) long-term results are poor in patients with a low preoperative EF. These findings are now being applied to patients followed in our OPD to determine whether echocardiographic assessment of changes in LV volume and EF provides a more sensitive means than the traditional clinical parameters to judge optimal time for operative intervention.

### Effects of Nitroglycerin on Exercise Capacity and on the Hemodynamic Response to Exercise in Patients with Valvular Heart Disease

Although the use of TNG has been traditionally reserved for patients with coronary artery disease, we have assessed the potential clinical utility of TNG in patients with valvular heart disease. Thus far, 9 patients have been studied, most with mitral and aortic valve lesions. Consistent increases in exercise tolerance and hemodynamic response to upright exercise has been documented. Our results suggest that vasodilator therapy may be a useful adjunct in the pharmacologic management of patients with valvular heart disease by reducing exertional symptoms and increasing exercise tolerance.

## SCINTIGRAPHY IN THE ASSESSMENT OF HEART DISEASE

Newly-developed scintigraphic techniques have the potential of revealing cardiac anatomic abnormalities and patterns of myocardial perfusion and motion that either are not available with more traditional angiographic techniques, or only can be obtained invasively. To determine the applicability of scintigraphic techniques to clinical cardiology, and to explore their limits in providing investigational information not otherwise obtainable, several

studies are in progress.

For example, it is generally accepted that coronary lesions producing 50% stenosis or less are of no functional significance; hence, patients with such lesions are not considered candidates for bypass surgery. Recent studies using a dual isotope technique to assess relative myocardial perfusion, however, suggest that inadequate perfusion after a vasodilatory stimulus can result from "subcritical" coronary lesions. We therefore are evaluating by intracoronary scintigraphic techniques the relative significance of coronary stenotic lesions of varying severity by determing adequacy of regional myocardial perfusion at rest and at the time of pacing-induced angina.

## MOLECULAR MECHANISMS RESPONSIBLE FOR CARDIAC CONTRACTION AND CELLULAR PROLIFERATION

The Section on Molecular Cardiology has conducted research in three areas: 1) phosphorylation of contractile proteins of the heart, 2) the effect of phosphorylation on platelet and other cellular myosins, 3) the function of contractile proteins in non-muscle cells.

1) Cardiac protein phosphorylation. We have found that a protein tentatively identified as M-protein, which is known to be located at the center of the myosin filament, can be phosphorylated with $\gamma$-labeled $AT^{32}P$. For these studies, canine cardiac myosin and surgical specimens from patients with asymmetric septal hypertrophy were utilized. The studies have shown: a) purified cardiac myosin contains protein(s) of molecular weight 150,000-160,000 which can be phosphorylated. b) This protein can be separated from myosin by Sepharose chromatography in a high ionic strength buffer. c) M-protein prepared from heart can be phosphorylated and appears to be the same protein we isolated that was bound to cardiac myosin. d) Present studies are directed toward positive identification of this protein as being derived from the M-band (utilizing antibody techniques) and uncovering the role of this phosphorylation in cardiac contraction.

2) Phosphorylation and actin-myosin interaction. We previously have found that the 20,000 dalton light chain of platelet myosin is phosphorylated. Recent studies in our laboratory have resulted in the purification of the enzyme from platelets that catalyze this phosphorylation.

We recently have shown that the effect of phosphorylation is to increase the actin-activated ATPase activity of platelet myosin by 5-8 fold. Dephosphorylation of previously phosphorylated platelet myosin by E. coli alkaline phosphatase results in a decrease in the actin-activated platelet myosin ATPase activity. The possibility that phosphorylation of myosin may serve as a switch for actin-myosin interaction in both non-muscle cells and smooth muscle cells is suggested by the finding that the platelet kinase can phosphorylate the 20,000 dalton light chain of mouse fibroblast (a non-muscle myosin) and chicken gizzard myosin (a smooth muscle myosin).

3) Myosin phosphorylation and cell proliferation. Non-muscle contractile proteins are thought to play a role in cell division, embryonic development and cell secretion. Myosin has been isolated from early myoblast cells prior

to cell fusion and found to have light chains similar to the myosin found in adult non-muscle cells. This suggests that the non-muscle type of myosin plays a role in muscle cells before they differentiate; i.e., before the gene for skeletal muscle light chains is turned on. Rhabdomyosarcoma cells are examples of a differentiated muscle cell that has de-differentiated by becoming a tumor cell. We have evidence suggesting that these cells also produce a non-muscle type of myosin. Hence, two cell types (myoblasts and rhabdomyosarcoma cells), which are known to divide at a much higher rate than normal muscle cells have been found to produce a myosin identical to non-muscle myosin. In addition, excessive proliferation of medial cells of the arterial wall have been implicated in the genesis of atherosclerosis. We therefore have initiated studies of the mechanism of medial cell proliferation; in particular, we are exploring the role of cytoplasmic myosin in cell division and the effect of phosphorylation in medial cells obtained at operation from patients with and patients without coronary artery disease.

Finally, Dr. Marshall Elzinga has sequenced three peptides from human platelet actin prepared in our laboratory and has compared the sequences to the amino acid sequence of rabbit skeletal muscle actin. Two of the peptides comprising 20 residues have the exact same sequence. One peptide of 9 residues has a single amino acid substition (threonive for valine). Further work, using human cardiac actin should answer the question as to whether this substitution is species specific (rabbit vs man) or is due to a difference in sequence between muscle and non-muscle (platelet) actin. Further sequence work on actin from human heart and human platelets should aid in uncovering differences in the structure and functions of these contractile proteins.

Project No. Z01 HL 01601-01 CB
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Effects of Altered Autonomic Innervation of the Heart on Ventricular Electrical Stability in Chronic Heart Failure

Previous Serial Number: None

Principal Investigator: Kenneth M. Kent, M.D.

Other Investigators: Kathleen Muth, B.S.
David M. Jacobowitz, Ph.D.
Stephen E. Epstein, M.D.

Cooperating Units: Laboratory of Clinical Science, NIMH

Project Description: The autonomic nervous system has profound influences on the electrical stability of ventricular myocardium. Moreover, the influences of the parasympathetic and sympathetic systems have opposite effects. Increased vagal tone decreases the propensity of the heart to develop ventricular fibrillation, while enhanced neural sympathetic tone increases the likelihood of developing ventricular fibrillation (VF). Since heart failure is known to alter cardiac neuronal stores of norepinephrine as well as to alter certain cardiovascular reflexes, we have studied the effects of heart failure on the electrical stability of the heart and on the cardiac responses to vagal stimulation.

An infrarenal aorto-caval anastomosis was performed on nine adult male dogs. An average of eight weeks later, the dogs developed cardiac failure. VF threshold was assessed in these animals and in a control group of dogs at constant heart rate and under pentobarbital anesthesia. Since barbiturates are vagolytic, this preparation allows for a relatively pure assessment of the effects exerted by differences in adrenergic tone.

Under these conditions, the VF threshold in the failure animals was $130\pm12$ mamp, a value significantly higher than in the control animals, $20\pm3$ mamp ($p<.001$). This enhanced electrical stability of the dogs in failure was associated with a 63% reduction of cardiac norepinephrine content. Decreased adrenergic innervation of the heart was confirmed by fluorescent microscopy.

To establish the causal role of norepinephrine depletion in elevating VF threshold, pharmacologic depletion of neural norepinephrine was accomplished with 6 hydroxydopamine in another group of animals, not in heart failure. VF threshold measured 5 days after administration of 6 hydroxydopamine, when cardiac norepinephrine was undetectable, was elevated to $88\pm15$ ma ($p<.01$), a

value similar to that obtained in the animals with chronic heart failure. Thus, depletion of cardiac neuronal norepinephrine, whether occurring as a consequence of chronic heart failure or as a result of pharmacologic intervention, results in an elevated VF threshold.

Cholinergic innervation of the heart and the physiologic effects of vagal stimulation were also studied in the heart failure preparation. Vagal stimulation decreased the atrial rate in a voltage dependent manner in each of the control animals and 7 of 9 of the failure animals. However, in two failure animals vagal stimulation caused no significant reduction in atrial rate. In these two dogs, cholinergic fibers, identified by specific stains for acetylcholinestinase were markedly reduced. Thus, in animals with heart failure, cholinergic innervation of the atrium may be diminished. Efferent vagal stimulation also increases VF threshold of the normal ventricle, an effect mediated by cholinergic fibers located in close proximity to the ventricular conducting system. These fibers were absent in two animals in heart failure, decreased in 5 and normal in 2. Moreover, VF threshold was essentially unaltered by vagal stimulation in 2 of 6 failure animals, both of which had reduced cholinergic innervation of the H-Purkinje system.

Thus, chronic cardiac failure leads to marked functional abnormalities in autonomic control of ventricular electrical stability. First, it appears that the well-described depression in cardiac norepinephrine stores contributes to an increase in the electrical stability of the heart. Such an observation is at variance with the commonly accepted belief that failure-induced depletion of cardiac norepinephrine is invariably deleterious, since it deprives the heart of one of its important compensatory mechanisms through which it can augment its depressed contractile state. Whether these two divergent effects of norepinephrine depletion--depressed contractile state and enhanced electrical stability--results in a net deleterious or salutary influence, is unknown. Second, cholinergic innervation of the ventricular septum was either reduced or absent in the majority of the hearts derived from failure animals, and in two of six animals, vagal stimulation did not raise VF threshold. Since vagal stimulation enhances ventricular electrical stability, deprivation of vagal influences by chronic heart failure would have a deleterious effect. Thus, failure-induced alterations in the adrenergic and cholinergic systems produce divergent effects on ventricular electrical stability. The relative magnitudes of each of these changes and the resultant interactions of the adrenergic and cholinergic systems may determine the likelihood of chronic hypertrophy and heart failure leading to arrhythmic death.

Keyword Descriptors: Heart Failure, Ventricular Fibrillation, Autonomic Nervous System, Adrenergic Innervation, Cholinergic Innervation

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Proposed Course of Project: Completed

Honors and Awards: None

Publications: None

Project No. Z01 HL01602-01 CB
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Enhanced Survival During Acute Myocardial Infarction in Reserpine Treated Dogs

Previous Serial Number: None

Principal Investigator: Richard A. Goldstein, M.D.

Other Investigators: Kenneth M. Kent, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: None

Project Description: Over half of all deaths from acute myocardial infarction occur early, before the patient obtains medical assistance. Currently, there are no preventive measures that can effectively reduce the frequency of sudden, presumably arrhythmic deaths.

Previous studies have demonstrated that the autonomic nervous system has important influences on the incidence of lethal ventricular arrhythmias in the early phase of experimental acute myocardial infarction. For example, both vagal stimulation and catecholamine depletion (the latter produced either surgically or pharmacologically) decrease the incidence of spontaneous ventricular fibrillation in experimental myocardial infarction. The purpose of the present investigation is to determine whether reserpine, a clinically useful catecholamine-depleting agent, increases survival during experimental myocardial infarction when it is given chronically in doses comparable to those given clinically.

Male mongrel dogs weighing between 21.4 and 31.3 kg were randomly assigned to one of three treatment groups: control (no treatment), low dose (0.1 mg im for 6-10 days - equal to an approximate adult human dose of 0.25 mg p.o. q.d.), and high dose (0.2 mg for 6-10 days). Animals were anesthetized with sodium pentobarbital (30 mg/kg) and intubated. The left anterior descending (LAD) and first septal coronary arteries were isolated through a left thoracotomy. After determining baseline heart rate and arterial and left atrial pressures, the heart was paced at 180 beats/min. The LAD and septal coronary arteries were then ligated and the animals were observed for 30 minutes or until ventricular fibrillation occurred. Biopsies of all four chambers of the heart were taken for norepinephrine determinations.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Survival times were 14.1 minutes for control animals, 16.1 minutes for low dose reserpine animals (NS) and 22 minutes for high dose reserpine dogs ($p<0.02$ high dose compared to control). Twenty-five percent of the control dogs, 22% of the low dose reserpine dogs and 53% of the high dose reserpine dogs survived the 30-minute observation period. In three animals receiving high dose reserpine in which ventricular fibrillation did not occur during the 30-minute observation period, observations were extended for an additional 70 minutes. There were no significant arrhythmias and no hemodynamic alterations during the longer observation period. Heart rate prior to pacing averaged 175 beats/minute for controls, 167 for low dose, and 138 for high dose reserpine ($p<.05$). Mean arterial pressure after 10 minutes of ischemia fell 3.4% in controls, 14% in low dose reserpinized animals, and 13.6% in high dose reserpine animals; there were no significant differences in the left atrial pressures in the three groups of animals during the observation period. Neuronal norepinephrine concentration in the left ventricle averaged 0.97 ug/g (1.28 to .75) in control animals, to 0.06 ug/g in the low dose and 0.08 ug/g in the high dose reserpine animals.

These data suggest that catecholamine depletion achieved with clinically employed doses of reserpine is protective against ventricular fibrillation following experimental myocardial infarction. If these trends are supported by studies in additional animals, clinical trials might be warranted to evaluate the potential antiarrhythmic actions of reserpine in man.

Keyword Descriptors: Acute Myocardial Infarction, Ventricular Fibrillation, Reserpine, Catecholamine Depletion, Sudden Death

Proposed Course of Project: Continuing

Honors and Awards: None

Publications: None

Project No. Z01 HL 01603-01 CB
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Cholinergic Enhancement of Ventricular Electrical Stability: Adrenergic Dependency or Primary Action?

Previous Serial Number: None

Principal Investigator: Kenneth M. Kent, M.D.

Other Investigators: Kathleen Muth, B.S.
Stephen E. Epstein, M.D.

Cooperating Units: None

Project Description: Increased vagal tone elevates ventricular fibrillation threshold, reduces the incidence of spontaneous ventricular fibrillation after coronary occlusion, and diminishes the incidence of digitalis toxic arrhythmias. The anatomic pathways that mediate these beneficial effects have been identified in the ventricular conducting system. In contrast, increased sympathetic neural stimulation decreases ventricular electrical stability. Since the cardiac effects of altering the activity of the adrenergic and cholinergic systems in many instances appears to be due to the interplay of one of these systems on the other, it has been postulated that the beneficial effects of increased vagal tone on ventricular electrical stability are due to the suppression of the effects of the sympathetic nervous system. To test this hypothesis, neuronal norepinephrine was depleted by the administration of 6 hydroxy dopamine in a group of six animals. Three to five days later, when cardiac norepinephrine was undetectable by chemical analysis, ventricular fibrillation threshold was determined. In control animals, VF threshold averaged 22 $\pm$ 6 mamp. VF threshold in the norepinephrine depleted animals was so high in 4 of the animals that ventricular fibrillation could not be precipitated despite currents of 120 mamps or more. In the two animals in which ventricular fibrillation could be induced electrically, vagal stimulation raised VF threshold from 75 to 95 mamp in one animal and from 65 to 85 mamp in the other. Propranolol (one mg/kg), administered to block the cardiac effects of circulating catecholamines, did not change ventricular fibrillation threshold caused by vagal stimulation. Myocardial ischemia was induced by occlusion of the coronary artery in the four treated animals in which VF could not be precipitated initially; VF threshold fell to measurable values in two animals. Vagal stimulation raised the VF threshold during ischemia from 28 to 38 mamp in one animal and from 90 to 110 mamp in the other. Propranolol, one mg/kg, decreased VF threshold in the first animal from 28 to 20 mamp, but did not impair the vagally mediated response; vagal

stimulation increased the VF threshold to 42 in this animal. Propranolol increased VF threshold in the second animal such that VF could not be induced electrically. Thus, these preliminary studies, performed in animals in which cardiac adrenergic influences (both intrinsic and circulating) were abolished, suggest that enhancement of ventricular electrical stability caused by the cholinergic system does not occur merely by antagonizing the influences of the adrenergic system. Rather, it appears that release of acetylcholine has direct electrophysiologic effects.

Keyword Descriptors: Ventricular Fibrillation, Acetylcholine, Autonomic Nervous System, Norepinephrine

Proposed Course of Project: Continuing

Honors and Awards: None

Publications: None

Project No. Z01 HL 01604-03 CB
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Clinical Characteristics of Asymmetric Septal Hypertrophy

Previous Serial Number: NHLI-136(c)

Principal Investigator: Walter L. Henry, M.D.

Other Investigators: Chester E. Clark, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: None

Project Description: Since the early descriptions of IHSS, patients have been described with features suggestive of the disease but in whom no resting or provocable left ventricular outflow obstruction could be demonstrated. These findings have been interpreted as indicating that IHSS is only one manifestation of a disease spectrum; i.e., hypertrophic cardiomyopathy in which obstruction may or may not occur. Recently, we have confirmed this hypothesis by using echocardiography to identify a specific anatomic abnormality, the presence of which is independent of outflow obstruction. Asymmetric septal hypertrophy (ASH), characterized by a ventricular septum at least 1.3 times as thick as the posterior-basal left ventricular free wall, was found in all patients whose disorder falls within the IHSS disease spectrum. One hundred patients with ASH were examined. Analysis of multiple clinical parameters failed to distinguish the nonobstructive ASH patients from the obstructive group with two exceptions: in nonobstructive ASH the murmur was softer with little variation following provocative maneuvers and a bisferious carotid pulse was absent. Angina and syncope, symptoms usually considered characteristic of obstruction, were also common in patients without obstruction. We conclude 1) obstructive and nonobstructive ASH patients cannot be distinguished symptomatically, 2) the absence of typical physical findings makes the clinical diagnosis of nonobstructive ASH difficult, and 3) echocardiography is the simplest and most reliable means of establishing the diagnosis in patients with ASH.

Keyword Descriptors: Obstructive ASH, Nonobstructive ASH, Hypertrophic Cardiomyopathy, IHSS

Proposed Course of Project: Continuing

Honors and Awards: None

Publications: None

Project No. Z01 HL 01605-01 CB
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Comparison of Two-Dimensional Echocardiographic Systems

Previous Serial Number: None

Principal Investigator: Walter L. Henry, M.D.

Other Investigators: David J. Sahn, M.D.
James M. Griffith, M.S.E.E.
Hugh D. Allen, M.D.
Stanley J. Goldberg, M.D.

Cooperating Units: Department of Pediatrics, University of Arizona Medical Center and Biomedical Engineering and Instrumentation Branch, DRS

Project Description: Real-time cross-sectional images of the heart were obtained in 44 patients with complex congenital heart disease using either a multiple crystal or a mechanical sector-scanner echocardiographic system. Congenital malformations studied included single ventricle (6), "corrected" transposition (8), d-transposition of great arteries (6), endocardial cushion defect (8), Ebstein's malformation (4), aortic stenosis (6), and ventricular septal defect (6). The multiple crystal system allowed a larger area of the heart to be visualized simultaneously and resulted in more rapid demonstration of the contour and positional relations of atrioventricular valves and great arteries. The mechanical sector-scanner visualized a smaller area of the heart simultaneously, but provided a higher resolution image that was particularly useful in analyzing the shape of great arteries and the insertion of atrioventricular valves. The current study indicates that these two echocardiographic systems provide complimentary information for the evaluation of complex congenital heart disease.

Keyword Descriptors: Two-Dimensional Echocardiography, Congenital Heart Disease, Sector-Scanner, Multiple Crystal Imaging

Proposed Course of Project: Completed

Honors and Awards: None

Publications: None

Project No. Z01 HL 01606-02 CB
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Echocardiographic Findings in Patients With Hypereosinophilia

Previous Serial No: NHLI-3(c)

Principal Investigator: Jeffrey S. Borer, M.D.

Other Investigators: Walter L. Henry, M.D.
David C. Dale, M.D.

Cooperating Units: National Institute of Allergies and Infectious Diseases

Project Description: Echocardiography (ECHO) is an increasingly important tool in the diagnosis and classification of primary and secondary cardiomyopathies. This report deals with echocardiographic evaluation of 8 men, aged 7 to 67, with chronic idiopathic hypereosinophilic syndromes (IHS). IHS had been present from 5 to 140 months. No patient was referred originally because of cardiac disease. Only one had clinical evidence of cardiac dysfunction. All 8 patients, with eosinophil counts ranging from 6900 to 94,000, had definite ECHO abnormalities. Most prominent was significant symmetrical thickening of the septum and left ventricular free wall, mean thickness being 14.3 mm $\pm$ 1.2 (SEM) (normal 9.4 mm $\pm$ .2, $p<.01$). The other 2 patients, with eosinophil counts of 3900 and 9500, had no abnormality but their septal and free wall thicknesses were at the upper limit of normal. Instantaneous left ventricular transverse dimension and velocity of circumferential fiber shortening were measured in every patient. No uniform abnormality in maximum velocity of circumferential fiber shortening was found. However, in 2 patients, 1 symptomatic, abnormalities in diastolic relaxation consistent with a restrictive defect were seen. The symptomatic patient also had transverse dimension slightly below the lower limit of normal. In about 1/3 of fatal idiopathic hypereosinophilic syndrome cases pathologic studies reveal endo- and myocardial fibrosis mural thrombi and ventricular hypertrophy with either constricted or dilated left ventricular cavities. Heretofore, it was believed that cardiac involvement in idiopathic hypereosinophilic syndrome leads rapidly to death. The present study suggests that ECHO may be of value in reassessing the prevalence and natural history of cardiac involvement in idiopathic hypereosinophilic syndromes. Moreover, ECHO may provide an objective parameter for evaluation of therapy in idiopathic hypereosinophilic syndromes.

Keyword Descriptors: Hypereosinophilia, Echocardiography, Cardiomyopathy

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Proposed Course of Project: Completed

Honors and Awards: None

Publications: Manuscript submitted to NEW ENGLAND JOURNAL OF MEDICINE

Project No. Z01 HL 01608-01 CB

1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Congenital Heart Disease Associated with ASH

Previous Serial Number: None

Principal Investigator: Barry J. Maron, M.D.

Other Investigators: Jesse E. Edwards, M.D.
Victor J. Ferrans, M.D., Ph.D.
Chester E. Clark, M.D.
Walter L. Henry, M.D.
Edward Lebowitz, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: Section of Pathology, NHLI

Project Description: ASH is characterized by a disproportionately thickened ventricular septum that contains numerous hypertrophied bizarrely-shaped and disorganized cardiac muscle cells. To determine whether such congenital cardiac malformations are part of the disease spectrum of genetically determined ASH, cardiac pathologic observations were made in eight patients with disproportionate septal thickening (ventricular septal to posterobasal left ventricular free wall thickness ratios of 1.5 to 2.5) and the following three categories of associated lesions: 1) parachute deformity of the mitral valve (occurring either as an isolated lesion or with ventricular septal defect, coarctation of the aorta, supravalvular ring of the left atrium, or double outlet right ventricle; 2) complete interruption of the aortic arch, and 3) ventricular septal defect. The arrangement of cardiac muscle cells in the disproportionately thickened ventricular septum was normal in six of the eight patients; in the other two patients (one with parachute deformity of the mitral valve and one with ventricular septal defect) numerous bundles of hypertrophied cardiac muscle cells were interlaced in a disorganized fashion among more normally arranged bundles of cells. First degree relatives of six of the eight patients were studied by echocardiography and found to have normal ventricular wall thicknesses and septal-free wall ratios.

It is concluded that disproportionate ventricular septal thickening may occur in patients with a variety of congenital heart malformations, but that such a finding is not necessarily a manifestation of the disease spectrum of genetically determined ASH.

Keyword Descriptors: Echocardiography, Hypertrophic Cardiomyopathy, Parachute Mitral Valve, Ventricular Septal Defect, Interruption Aortic Arch,

Project No. Z01 HL 01608-01 CB

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Coarctation of Aorta, Double Outlet Right Ventricle.

Proposed Course of Project: Completed

Honors and Awards: None

Publications: None

Project No. Z01 HL 01613-01 CB
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Measurement of Mitral Orifice Area by Two-Dimensional Echocardiography

Previous Serial Number: None

Principal Investigator: Walter L. Henry, M.D.

Other Investigators: James M. Griffith, M.S.E.E.
Lawrence L. Michaelis, M.D.
Charles L. McIntosh, M.D.
Andrew G. Morrow, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: Clinic of Surgery and Biomedical Engineering and Instrumentation Branch, Division of Research Services

Project Description: A quantitative assessment of mitral valve orifice area can be achieved in patients with pure mitral stenosis by cardiac catheterization. In the presence of mitral regurgitation, however, accurate measurement often is impossible because total diastolic flow through the mitral valve frequently is unknown. Using a recently developed real-time, two-dimensional echocardiography system, we were able to obtain a cross-sectional image of the mitral valve by scanning the heart perpendicular to its long axis at the level of the tip of the mitral leaflets. Twenty consecutive patients undergoing operation for mitral valve disease were studied during the week prior to operation. In 18 of 20 (90%) the mitral orifice was imaged successfully in early diastole by two-dimensional echocardiography so that mitral valve orifice area could be measured directly in square centimeters. In 14 patients (10 with associated mitral regurgitation), mitral orifice area was measured both by echocardiography and directly at time of operation. In 12 of 14 (86%) patients, mitral orifice area by two-dimensional echocardiography was within 0.3 square centimeters of that measured at operation (correlation coefficient for all 14 patients = 0.92; $p<0.01$). In conclusion the present study demonstrates that two-dimensional echocardiography is extremely useful in the evaluation of patients with mitral valve disease because it provides a noninvasive method for directly measuring the mitral valve orifice area that is accurate even in the presence of mitral regurgitation.

Keyword Descriptors: Mitral Valve Orifice, Mitral Stenosis, Two-Dimensional Echocardiography

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Proposed Course of Project: Completed

Honors and Awards: None

Publications: Manuscript to be published in CIRCULATION (May, 1975)

Project No. Z01 HL 01614-01 CB

1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Mechanism of Beneficial Action of TNG-Methoxamine in AMI

Previous Serial No: None

Principal Investigator: Howard J. Smith, MB. ChB.MRACP

Other Investigators: Richard A. Goldstein, M.D.
Kenneth M. Kent, M.D., Ph.D.
Roger Aamodt, Ph.D.
Stephen E. Epstein, M.D.

Cooperating Units: Department of Nuclear Medicine, NIH

Project Description: Previous reports from this laboratory have shown that treatment with nitroglycerin (TNG) following coronary artery occlusion in dogs has beneficial effects on infarct size and on the threshhold at which electrical stimulation induces ventricular fibrillation. These actions are potentiated when methoxamine is administered to abolish the TNG-induced fall in arterial pressure and reflex tachycardia. To elucidate the mode of action of TNG and methoxamine, we measured their effects on myocardial blood flow (MBF) and on ischemic injury during coronary occlusion.

Mongrel dogs with chronically implanted myocardial electrodes, left atrial catheters, and coronary occlusive cuffs were sedated with morphine and diazepam. MBF was measured in ischemic and non-ischemic myocardium by use of radioactive microspheres (15 $\pm$ 5$\mu$, labelled with $^{141}Ce$, $^{169}Yb$ and $^{85}Sr$). MBF to the center of the ischemic area was taken to represent collateral blood flow; MBF to the non-ischemic myocardium was used as an index of $MVO_2$. Ischemic injury was estimated by summating ST elevations ($\Sigma$ST) recorded from the myocardial electrodes.

MBF at the center of the infarct in 14 dogs averaged 20% of that present in non-ischemic regions. In 9 of the 14, collateral flow increased to 33% following 30 minutes of TNG administration. The enhanced collateral flow was associated in 7 dogs with a decrease in ST segment elevation; ischemia was either unchanged or increased in the other 2 dogs. In the 2 animals in which ST segment elevation did not diminish, TNG produced excessive tachycardia and did not reduce estimated $MVO_2$. Of the remaining 5 dogs collateral flow was unchanged by TNG in one and ST segments diminished only minimally; collateral flow diminished following TNG in 4 and ST segments were essentially unchanged (3 dogs) or increased (1 dog).

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In 7 of the 14 animals that received TNG, addition of methoxamine 20 minutes after the start of TNG infusion returned heart rate towards normal, but caused no consistent change in collateral flow, or $MVO_2$. However, methoxamine uniformly diminished ischemic injury in the dogs that were not responsive to TNG, mainly by reducing heart rate (and $MVO_2$), but also by restoring blood pressure in those dogs that had developed hypotension. Similar results were found when individual zones of myocardium were analyzed: methoxamine reduced ischemic injury when prior treatment with TNG was associated with excessive tachycardia or hypotension, but produced little change in those regions that had responded favorably to TNG.

In summary, the results of this investigation demonstrate that the salutary effects of TNG on ischemic injury during acute myocardial infarction are mediated by an increase in collateral flow and a reduction in $MVO_2$. However, if TNG causes hypotension (with resulting diminution in collateral flow) or excessive tachycardia (with resulting increase in $MVO_2$ and perhaps decrease in flow) reduction of ischemic injury will not occur without addition of methoxamine to reverse the TNG-induced pressure and heart rate changes.

Keywords Descriptors: Nitroglycerin, Myocardial blood flow, Collateral flow, Acute myocardial infarction, Methoxamine.

Proposed Course of Project: Continuing

Honors and Awards: None

Publications: None

Project No. Z01 HL 01615-04 CB
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Factors Affecting the Operative Mortality in Aortic Valvular Disease

Previous Serial Number: NHLI-18(c)

Principal Investigator: Walter L. Henry, M.D.

Other Investigators: Chester E. Clark, M.D.
Robert E. Golstein, M.D.
Samuel B. Itscoitz, M.D.
David R. Redwood, M.D.
D. Luke Glancy, M.D.
Alan S. Pearlman, M.D.
Joel Morganroth, M.D.
Stephen E. Epstein, M.D.
Andrew G. Morrow, M.D.
Larry Michaelis, M.D.
Charles L. McIntosh, M.D.

Cooperating Units: Clinic of Surgery, NHLI

Project Description: The purpose of this study is to define prospectively those preoperative factors that indicate an increased operative risk or that irreversible myocardial dysfunction has occurred. All patients 18 years old or over admitted to the Cardiology Branch for aortic valve replacement are being evaluated. Patients with significant involvement of other valves are excluded. Preoperative and 6-month postoperative assessment are based mainly on data obtained by cardiac catheterization (including coronary arteriography) and echocardiography. Evaluation of myocardial function includes ventricular volumes, LV mass, mean dv/dt, mean VCF, and ejection fraction. These data, as well as operative risk, will also be correlated with EKG, x-ray, phonocardiogram, and exercise testing.

Seventy-eight patients have been assessed preoperatively. Fifty-five of the 78 have been studied also at the 6-month postoperative assessment point. Preliminary results suggest that many patients who in the past would not have been offered surgery because of a suspected high risk, in fact do well following operation.

Keyword Descriptors: Aortic Regurgitation, Operative Mortality, Echocardiography, Aortic Stenosis

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Individual Project Report
July 1, 1974 through June 30, 1975

Proposed Course of Project: Continuing

Honors and Awards: None

Publications: None

Project No. Z01 HL 01617-01 CB
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Differential Diagnosis of Great Artery Anomalies by Two-Dimensional Echocardiography

Previous Serial Number: None

Principal Investigator: Walter L. Henry, M.D.

Other Investigators: Barry J. Maron, M.D.
James M. Griffith, M.S.E.E.
David R. Redwood, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: Biomedical Engineering and Instrumentation Branch, Division of Research Services

Project Description: A recently developed sector-scanner that produces real-time, two-dimensional echocardiograms was used to determine whether visualization of the great arteries at their origin would provide diagnostically useful information in patients with cyanotic heart disease. Thirty-one patients (age 2 to 31 years; weight 5 to 50 kg) previously diagnosed by cardiac catheterization were studied. Images were generated perpendicular to the long axis of the heart at the level of the origin of the great arteries. Satisfactory great artery visualization was obtained in 27 of 31 patients. Arteries in cross-section appeared as circles; those sectioned longitudinally appeared sausage-shaped. Three great artery patterns were seen: a) a large single circle, seen in four patients with truncus arteriosus and two with pulmonary atresia; b) two adjacent circles, seen in 11 patients with transposition of great arteries, two patients with corrected transposition and one with double outlet right ventricle; and c) a circle with a sausage-shaped structure curving anteriorly from right to left, seen in five patients with tetralogy of Fallot, one patient with a large ventricular septal defect complicated by an Eisenmenger reaction and one patient with a ventricular septal defect and valvular pulmonic stenosis. This latter pattern, also seen in ten normal subjects, is characteristic of normally related great arteries. We conclude that two-dimensional echocardiography is an accurate noninvasive technique for categorizing individuals with congenital anomalies of the great arteries.

Keyword Descriptors: Great Artery Anomalies, Two-Dimensional Echocardiography, Transposition of Great Arteries, Truncus Arteriosus, Tetralogy of Fallot

Proposed Course of Project: Completed

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Honors and Awards: None

Publications: Manuscript published in CIRCULATION 51:283-291, 1975

Project No. Z01 HL 01618-03 CB
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Long-Term Effects of Operation on Obstruction and LV Hypertrophy in IHSS

Previous Serial Number: NHLI-21(c)

Principal Investigator: Walter L. Henry, M.D.

Other Investigators: Stephen E. Epstein, M.D.
Chester E. Clark, M.D.
Andrew G. Morrow, M.D.

Cooperating Units: Clinic of Surgery, NHLI

Project Description: Surgical myectomy for IHSS results in symptomatic improvement and loss of LV outflow obstruction. To determine the mechanism by which the obstruction disappears, and the long-term effects of operation, we used echocardiography to measure mitral valve position, mitral valve systolic motion, and left ventricular free wall thickness in two groups of patients with IHSS: 13 patients who had myectomy performed 2 to 11 years (mean 6.5 years) previously, and 27 nonoperated patients. Preoperative hemodynamic data were comparable in both groups. The prominent forward mitral valve motion in midsystole, indicative of obstruction, was present in each nonoperated and absent in each operated patient. Mitral valve position at onset of systole was determined by calculating the ratio of mitral valve-posterior left ventricular wall distance to septal-mitral valve distance. In normals, the mitral valve is positioned near the posterior left ventricular wall (ratio 0.28$\pm$.01). While the mitral valve is anteriorly positioned in both IHSS groups, it is more anterior in nonoperated patients (ratio 1.04$\pm$ 106) than in operated patients (ratio .66$\pm$.04, $p<.01$). Intraoperative studies in six patients revealed the mitral valve to assume a more posterior position immediately after myectomy; this coincided with disappearance of the midsystolic forward mitral valve movement. Left ventricular free wall thickness was 13.2$\pm$.05 mm in the nonoperated patients (normal 9.4$\pm$.02), and 11.5$\pm$.04 mm in the operated ($p<.05$). We conclude the mitral valve is tethered forward in IHSS. Septal myectomy relieves this tethering and thereby abolishes the mid-systolic forward mitral valve motion and hence left ventricular outflow obstruction. Abnormal mitral valve position and motion did not recur postoperatively during long-term follow-up. Finally, left ventricular free wall thickening appears to regress postoperatively.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Keyword Descriptors: Myotomy, Myectomy, IHSS, Obstructive ASH, Echocardiography

Proposed Course of Project: Continuing

Honors and Awards: None

Publications: None

Project No. Z01 HL 01619-04 CB
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Distribution of the Cardiomyopathy in IHSS

Previous Serial Number: NHLI-22(c)

Principal Investigator: Walter L. Henry, M.D.

Other Investigators: Chester E. Clark, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: None

Project Description: Patients with typical idiopathic hypertrophic subaortic stenosis (IHSS) represent only one subgroup of a cardiac disease in which the characteristic anatomic abnormality is asymmetric septal hypertrophy (ASH). In most patients with ASH, left ventricular outflow obstruction is absent and cardiac dysfunction presumably is due to widespread involvement of the left ventricle by an underlying myocardial abnormality. In other patients with ASH, left ventricular outflow obstruction is present (typical IHSS) and constitutes a major feature of the hemodynamic and physical findings. To determine whether patients with outflow obstruction also have the underlying myocardial abnormality diffusely involving the left ventricle, the gross morphology of hearts from patients with and without outflow obsturction were studied both by necropsy and by echocardiography. Echocardiographic studies revealed that the ventricular septum was thicker in obstructive ASH, a finding confirmed by the postmortem studies. The necropsy studies also indicated that although the left ventricular free wall was thickened in both obstructive and nonobstructive ASH, the configuration of the left ventricular free wall was distinctly different in the two groups. In obstructive ASH, the free wall was hypertrophied and identical in appearance to that seen in valvular aortic stenosis. Moreover, echocardiographic studies indicated that the thickening of the free wall behind posterior mitral leaflet appeared to regress after operative relief of the outflow obstruction. In contrast, the left ventricular free wall of severely symptomatic patients without outflow obstruction had a markedly different and unique appearance; the free wall of left ventricle directly behind the posterior mitral leaflet was of normal or less than normal thickness, whereas the remaining free wall was nonuniformly thickened. On the basis of these findings and the microscopic data presented in the companion paper, we conclude that the myocardial abnormality in obstructive ASH (typical IHSS) is localized largely to the ventricular septum, with left ventricular free wall thickening occurring as a consequence of outflow



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obstruction. In symptomatic patients with nonobstructive ASH, however, the data suggest that the left ventricle, including free wall, is extensively involved with a primary myocardial abnormality.

Keyword Descriptors: Asymmetric Septal Hypertrophy, Hypertrophic Cardiomyopathy, Necropsy, Echocardiography, IHSS

Proposed Course of Project: Completed

Honors and Awards: None

Publications: CIRCULATION 50:447-455, 1974

Project No. Z01 HL 01620-01 CB
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Pathophysiology and Prediction of Onset of Atrial Fibrillation

Previous Serial No: None

Principal Investigator: Walter L. Henry, M.D.

Other Investigators: Joel Morganroth, M.D.
Alan S. Pearlman, M.D.
Chester E. Clark, M.D.
David R. Redwood, M.D.
Samuel B. Itscoitz, M.D.
Stephen E. Epstein, M.D.

Project Description: In an attempt to more completely understand the pathophysiology of atrial fibrillation, echocardiography was used to study 85 patients with isolated mitral valve disease, 50 patients with isolated aortic valve disease, and 130 patients with asymmetric septal hypertrophy. In all three groups of patients, atrial fibrillation was common only in the subgroup of patients older than 40 years of age who in addition had an echocardiographically measured left atrial transverse dimension exceeding 45mm. Mean left atrial pressure measured at cardiac catheterization did not provide nearly as strong a predictive index of atrial fibrillation. The results of the present study suggest that a chronic hemodynamic burden initially produces left atrial enlargement which in turn predisposes to atrial fibrillation. Of clinical importance, atrial fibrillation was rare in patients with a left atrial transverse dimension below 40mm (3 of 117 or 3%) but common when this dimension exceeded 40mm (80 of 148 or 54%). Because 10 of 81 (12%) patients in the present series had an embolus at the onset of atrial fibrillation, it appears reasonable to consider anticoagulation and antiarrhythmic therapy in the management of a patient in normal sinus rhythm who has a left atrial transverse dimension exceeding 40mm. Observations of left atrial size in patients in whom cardioversion was attempted suggest that successful cardioversion is uncommon when left atrial transverse dimension exceeds 45mm. Moreover, in patients with asymmetric septal hypertrophy who have a left atrial transverse dimension exceeding 50mm, the risk of cardioversion-induced embolization may well be greater than the likelihood of achieving stable sinus rhythm.

Keyword Descriptors: Atrial Fibrillation, Systemic Embolus, Left Atrial Size, Cardioversion, Echocardiography

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Proposed Course of Project: Completed

Honors and Awards: None

Publications: Manuscript submitted to CIRCULATION

Project No. Z01 HL 01621-03 CB
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: A Real Time System for Two-Dimensional Echocardiography

Previous Serial Number: NHLI-26(c)

Principal Investigator: Walter L. Henry, M.D.

Other Investigators: James M. Griffith, M.S.E.E.

Cooperating Units: Biomedical Engineering and Instrumentation Branch, DRS

Project Description: During the past several years one-dimensional pulse-echo ultrasound techniques have proven extremely useful in cardiac diagnosis. A one-dimensional system, however, only visualizes structures lying along a single straight line. The spatial relationships of the various cardiac structures are therefore not so easily defined as with two-dimensional systems which display the heart by constructing a plane image composed of many straight lines. We have developed a sector scanning system for obtaining two-dimensional echocardiograms in real time using ultrasonic pulse-echo techniques. Images are produced by angling rapidly a single transducer through a 30-degree sector from a fixed spot (between ribs) on the patient's chest. Thirty complete sectors (or frames) are produced per second. The use of a large diameter transducer ensures that signal strength is good and cardiac structures, including endocardium, can be visualized. Other advantages include high transducer sensitivity, real time imaging and easy visualization of various regions of the heart. Experience with more than 100 patients indicates that diagnostic quality two-dimensional echocardiograms can be readily obtained in essentially the same patients from whom one-dimensional echocardiograms are recorded and can usually be performed in less time.

Keyword Descriptors: Two-Dimensional Echocardiography, Mechanical Sector-Scanner, Real-Time Imaging

Proposed Course of Project: Completed

Honors and Awards: None

Publications: CIRCULATION 49:1147-1152, 1974

Project No. Z01 HL 01622-01 CB
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Effects of Space Flight on Cardiac Function

Previous Serial Number: None

Principal Investigator: Walter L. Henry, M.D.

Other Investigators: Stephen E. Epstein, M.D.
James M. Griffith, M.S.E.E.
Robert E. Goldstein, M.D.
David R. Redwood, M.D.

Cooperating Units: Biomedical Engineering and Instrumentation Branch, DRS

Project Description: Echocardiographic studies were performed preflight five days before lauch and on recovery day and 1, 2, 4, 11, 31 and 68 days postflight. From these echocardiograms, the following measurements were made: 1) left ventricular transverse dimension at end-diastole, 2) left ventricular transverse dimension at end-systole, and 3) ventricular free wall thickness at end-diastole. From these primary measurements, left ventricular end-diastolic volume, end-systolic volume, stroke volume, and mass were derived using the accepted assumptions. Preflight measurements in the Commander revealed the left ventricular end-diastolic volume, stroke volume, and mass to be at the upper limit of normal, while those of the Scientist Pilot and Pilot were increased significantly above the normal range. These findings findings in the Scientist Pilot and Pilot resemble those seen in trained distance runners. Wall thickness measurements were normal in all three crewmembers preflight. Postflight basal studies were unchanged in the Commander on recovery day through 68 days postflight. In both the Scientist Pilot and Pilot, however, the left ventricular end-diastolic volume, stroke volume, and mass were decreased slightly. These decreases were noted on recovery day through 11 days postflight but had returned to near normal by 31 days postflight. Wall thickness measurements were unchanged. Left ventricular function curves were constructed for the Commander and Pilot by plotting stroke volume versus end-diastolic volume. In both astronauts, preflight and postflight data fell on the same straight line demonstrating that no deterioration in cardiac function had occurred. These data indicate that the cardiovascular system adapts well to prolonged weightlessness and suggest that alterations in cardiac dimensions and function are unlikely to limit man's future in space.

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Keyword Descriptors: Space Flight, Cardiac Function, Skylab 4 Astronauts, Echocardiography

Proposed Course of Project: Completed

Honors and Awards: None

Publications: Manuscript published in PROCEEDINGS OF SKYLAB LIFE SCIENCES SYMPOSIUM (August 27-29, 1974), pp. 711-721

Project No. Z01 HL 01623-03 CB
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Mitral Valve Position in Patients with ASH

Previous Serial Number: NHLI-19(c)

Principal Investigator: Walter L. Henry, M.D.

Other Investigators: Chester E. Clark, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: None

Project Description: Left ventricular outflow obstruction in patients with IHSS or obstructive asymmetric septal hypertrophy (ASH) is due to abnormal forward motion during systole of the anterior mitral leaflet. In order to determine why some patients with ASH have left ventricular outflow obstruction while others do not, we studied a large number of patients with ASH using both one and two dimensional echocardiography. In 100 patients with ASH and 22 normal subjects, mitral valve position at onset of systole was quantitated by measuring the distance from the ventricular septum to the mitral valve and the distance from the mitral valve to the posterior left ventricular wall. None of the normal subjects and only 3 of 51 non-obstructive ASH patients (6%) had a septal-mitral valve distance less than 20 mm compared to 23 of 35 obstructive ASH patients (66%). Moreover, in ASH the mitral valve at onset of systole was actually positioned forward in the left ventricular cavity. Two-dimensional studies in 11 patients with obstructive ASH revealed that contraction of the malaligned papillary muscles did not cause the abnormal forward mitral valve motion. We propose that the left ventricular outflow obstruction in patients with obstructive ASH occurs as a result of two factors: 1) narrowing of the left ventricular outflow tract at onset of systole, and 2) hydrodynamic forces generated by contraction of the left ventricle.

Keyword Descriptors: Obstructive ASH, IHSS, Outflow Obstruction, Echocardiography, Two-Dimensional Imaging

Proposed Course of Project: Completed

Honors and Awards: None

Publications: AMERICAN JOURNAL OF CARDIOLOGY 35:337-345, 1975

Project No. Z01 HL 01624-01 CB
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Aortic Regurgitation: Cardiac Function and Operative Result

Previous Serial Number: None

Principal Investigator: Walter L. Henry, M.D.

Other Investigators: Joel Morganroth, M.D.
Chester E. Clark, M.D.
Alan S. Pearlman, M.D.
Leonard Grauer, M.D.
David R. Redwood, M.D.
Samuel B. Itscoitz, M.D.
Charles L. McIntosh, M.D.
Lawrence L. Michaelis, M.D.
Andrew G. Morrow, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: Clinic of Surgery, NHLI

Project Description: To assess the influence of ventricular function on operative results in patients with isolated aortic regurgitation, 20 patients undergoing aortic valve replacement were studied echocardiographically preoperatively and again six months postoperatively. Patients were subdivided into three groups based on preoperative ejection fraction (EF): normal EF (>60%)-11 patients; intermediate EF (40-60%)-5 patients; low EF (<40%)-4 patients. Two operative deaths occurred, both in patients with intermediate EF. EF did not increase after operation in any patient. In two patients with normal EF preoperatively, EF decreased; both experienced operative complications. Of nine patients with normal EF both preoperatively and postoperatively, eight had reduction in end-diastolic volume (LVEDV) postoperatively to <250 ml, 7 a decrease in LV mass to <500 g; all are functional class I or II. Of patients with either intermediate EF both preoperatively and postoperative (3 patients), or normal EF preoperatively but intermediate EF postoperatively (1 patient), 3 of 4 have LVEDV postoperatively <250 ml, 4 of 4 have LV mass <500 g; 3 of 4 are class 1 or II. In contrast, no patient with either low EF both preoperatively and postoperatively (4 patients) or normal EF preoperatively but low EF postoperatively (one patient) had LVEDV <250 ml or LV mass <500 g. Three of the five died during long-term follow-up; one is class III and one class II. Thus, in patients with aortic regurgitation 1) operative does not improve vasal ventricular function, 2) LVEDV and mass are more likely to return toward normal in patients with normal or intermediate EF, 3) long-term results are

good in patients with normal or intermediate EF provided an operative complication does not impair ventricular function, 4) long-term results are poor in patients either with a low preopeartive EF or in whom an operative complication results in a low EF postoperatively.

Keyword Descriptors: Echocardiography, Aortic Regurgitation, Ejection Fraction, Operative Results

Proposed Course of Project: Continuing

Honors and Awards: None

Publications: Abstract published in CLINICAL RESEARCH, April, 1975

Project No. Z01 HL 01625-02 CB
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

NIH-PHS
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Identification of Cyanotic Heart Disease in Infants by Two-Dimensional Echocardiography

Previous Serial Number: NHLI-128(c)

Principal Investigator: Barry J. Maron, M.D.

Other Investigators: Walter L. Henry, M.D.
Robert Freedom, M.D.
David T. Kelly, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: Department of Pediatrics, Johns Hopkins Hospital, Baltimore, Maryland

Project Description: Real-time, two-dimensional echocardiography was used to identify great artery relations in 23 infants and small children, including 16 patients with angiographically documented transposition of the great arteries, tetralogy of Fallot, or pulmonary atresia. Using this tec-nique, the heart was scanned perpendicular to its long axis at the origin of the great arteries. Great arteries cross-sectioned perpendicular to their long-axes appears as circles; when sectioned longitudinally, these arteries appeared as elongated, sausage-shaped structures. In patients with normally related great arteries, a circular structure (aorta) always was positioned posterior to an elongated, sausage-shaped structure (distal right ventricular outflow tract and proximal main pulmonary artery). In transposition of the great arteries, two adjacent circular structures were observed; the anterior circle (aorta) was located either to the right, left or directly anterior to the posterior circle (pulmonary artery). In pulmonary atresia or hypoplasia, a large posterior circle (aorta) was associated with an anteriorly positioned structure that was either short and small (atretic right ventricular outflow tract) or elongated with an area of severe narrowing (hypoplastic right ventricular outflow tract).

Keyword Descriptors: Transposition of the Great Vessels, Tetralogy of Fallot, Pulmonary Atresia

Proposed Course of Project: Completed

Honors and Awards: None

Publications: None

Project No. Z01 HL 01626-01 CB
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Intramitochondrial Glycogen Deposits in Cardiac Muscle

Previous Serial Number: None

Principal Investigator: Barry J. Maron, M.D.

Other Investigators: Victor J. Ferrans, M.D., Ph.D.

Cooperating Units: Section of Pathology, NHLI

Project Description: Intramitochondrial glycogen deposits were present in ventricular muscle cells in 4 of 16 patients with aortic valvular disease and 5 of 16 patients with asymmetric septal hypertrophy. The intramitochondrial glycogen deposits were located in the outer mitochondrial compartment (intracristal space) in each instance and were of the monoparticulate (β) type in 7 patients; both β-glycogen and α-glycogen rosettes were present in mitochondria from the other 2 patients. Mitochondria containing glycogen were present in about equal frequency in hypertrophied cells with otherwise normal ultrastructure and in cells with features of degeneration. Intramitochondrial glycogen appears to be a relatively common finding in hypertrophied myocardium in a variety of cardiac conditions.

Keyword Descriptors: Myocardial Ultrastructure, Aortic Valvular Disease, Asymmetric Septal Hypertrophy, Hypertrophic Cardiomyopathy, Cardiac Muscle Cells, Electron Microscopy

Proposed Course of Project: Completed

Honors and Awards: None

Publications: None

Project No. Z01 HL 01627-01 CB
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

NIH-PHS
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Phosphorylation of Cardiac Muscle Proteins

Previous Serial Number: None

Principal Investigator: Barry J. Maron, M.D.

Other Investigators: Robert S. Adelstein, M.D.

Cooperating Units: None

Project Description: Phosphorylation of cardiac muscle proteins is a potentially important mechanism in regulating the interaction of actin and myosin during contraction. In order to determine the characteristics of phosphorylation in the heart, crude preparations of canine and human cardiac actomyosin were incubated with $\gamma$-labeled $AT^{32}P$. Incorporation of $^{32}P$ into two proteins was demonstrated: 1) a protein with a molecular weight of 165,000 daltons that was identified as M-protein, and 2) the light chain of mysoin having a molecular weight of 27,000 daltons in the dog and 25,000 daltons in man. In addition, incubation of $AT^{32}P$ with preparations of M-protein produced equal incorporation of $^{32}P$ into two separate protein components (each having a molecular weight of about 165,000 daltons). The phosphorylation of M-protein and of a light chain of myosin appears to be due to an endogeneous kinase or kinases.

Keyword Descriptors: M-Protein, Myosin, Light Chain of Myosin, Protein Kinase

Proposed Course of Project: Continuing

Honors and Awards: None

Publications: None

Project No. Z01 HL 01628-02 CB
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Sudden Infant Death Syndrome: Potential Cardiac Mechanisms

Previous Serial Number: NHLI-131(c)

Principal Investigator: Barry J. Maron, M.D.

Other Investigators: Chester E. Clark, M.D.
Robert E. Goldstein, M.D.
Walter L. Henry, M.D.
Russell B. Fisher, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: Coroner's Office, State of Maryland, Baltimore, Maryland

Project Description: Sudden infant death syndrome (SIDS) is a major cause of mortality in the first six months of life, but the primary mechanisms responsible for this condition are unknown. To investigate possible cardiac mechanisms, 42 sets of parents (who had at least one infant die of SIDS) were studied by echocardiography and electrocardiography. Asymmetric septal hypertrophy (ASH) was present in two (5%) parental sets (one parent affected in each set). At least one member of 13 (31%) other parental sets had ECG abnormalities; 9 with QT interval prolongation, two with left anterior hemiblock, one with first degree A-V block and one with ST-T abnormalities. To further study the role of QT interval prolongation in SIDS, two other avenues of investigation were pursued. First, QT intervals were assessed in siblings of infants dying suddenly of SIDS (in the nine families in which at least one member of the parental set showed QT interval prolongation). Thirteen of 27 (47%) of these siblings showed QT interval prolongation consistent with an autosomal dominant pattern of inheritance; the remainder had normal QT intervals. Second, three infants with "near-miss SIDS" were studied. One of these infants showed a markedly prolonged QT interval and the other two showed slightly prolonged QT intervals. In addition, histologic sections of myocardium were analyzed in another group of 45 infants with SIDS, 17 control infants who died suddenly from other causes, and 5 normal human fetuses. Small foci of normal-sized, disorganized myocardial cells were present in the ventricular septum of 10 (22%) of the infants with SIDS, one (6%) of controls and none of the fetuses. The foci of disorganized cells in SIDS resembled those in ASH, but were less marked in extent and severity. Although the significance of these abnormally arranged cells is unknown, they may serve as a nidus for ventricular arrhythmias. Furthermore, we studied five parental sets of these infants with disorganized cells; three had ASH and two of these had QT interval prolongation. Thus, the results of the

present study demonstrate that 30% of 47 parent sets of infants with SIDS had QT prolongation or ASH. Both these abnormalities have an autosomal dominant pattern of inheritance and have been associated with sudden death in children. Therefore, our data suggest that cardiac mechanisms may play a role in a considerable proportion of infant deaths falling within the sudden infant death syndrome.

Keyword Descriptors: QT Interval Prolongation, Asymmetric Septal Hypertrophy, Electrocardiography, Echocardiography

Proposed Course of Project: Completed

Honors and Awards: None

Publications: None

Project No. Z01 HL 01629-02 CB
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Asymmetric Septal Hypertrophy in Childhood

Previous Serial Number: NHLI-127(c)

Principal Investigator: Barry J. Maron, M.D.

Other Investigators: Walter L. Henry, M.D.
David R. Redwood, M.D.
Chester E. Clark, M.D.
William C. Roberts, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: Section of Pathology, NHLI

Project Description: Although considerable information is available concerning the clinical features and natural history of asymmetric septal hypertrophy (ASH) in adults, little is known of this disease in children. The clinical characteristics and course of 46 children with ASH, who were evaluated at the National Heart and Lung Institute, have been analyzed. Twenty-four children had obstruction to ventricular outflow; 22 children had no obstruction to ventricular outflow, including 11 patients without overt manifestations of cardiac disease other than echocardiographic evidence of ASH. Thirty-five of the 46 children have been followed for one to 16 years (average, 7.4 years). These latter children represent that subgroup of patients with ASH referred to the National Heart and Lung Institute and diagnosed prior to the general availability of echocardiography. The clinical course of these patients was variable. Eighteen (52%) of the 35 patients improved or remained stable, including two patients who underwent left ventricular myotomy-myectomy or myotomy and six patients who received propranolol. Six (17%) of the 35 patients deteriorated clinically and 11 (31%) of the 35 patients died suddenly (4% mortality per year). Two of the patients who died suddenly had previously undergone operation (six and 13 years previously) with resultant abolition of the outflow gradient; four others were taking propranolol. Neither symptmatology, electrocardiographic abnormalities, heart size, left ventricular ejection or upstroke time, magnitude of outflow gradient, or left ventricular end-diastolic pressure proved predictive of sudden death. Excluding patients who had previous operation, eight (40%) of 20 patients with obstruction who were followed long-term and one (9%) of 11 patients without outflow obstruction died suddenly. Thus, this study demonstrates that the clinical and hemodynamic spectrum of ASH in childhood is broad and sudden death has been relatively common in that subgroup of children who were referred to the National Heart and Lung

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Institute because of overt manifestations of cardiac disease.

Keyword Descriptors: Sudden Death, Hypertrophic Cardiomyopathy, Idiopathic Hypertrophic Subaortic Stenosis, Congenital Heart Disease, Echocardiography, Cardiac Catheterization, Myocardium, ASH, Childhood

Proposed Course of Project: Completed

Honors and Awards: None

Publications: None

Project No. Z01 HL 01630-01 CB
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Nitroglycerin, Nitroprusside and Myocardial Ischemia

Previous Serial Number: None

Principal Investigator: Alan S. Pearlman, M.D.
Robert Engler, M.D.

Other Investigators: Kenneth M. Kent, M.D., Ph.D.
Stephen E. Epstein, M.D.

Cooperating Units: None

Project Description: Recent studies have generated considerable enthusiasm for the use of the nitroglycerin (TNG) and nitroprusside in the treatment of patients with acute myocardial infarction. These agents appear to improve hemodynamic function by their peripheral dilator effects on systemic arteries and veins, which reduce arterial and left ventricular filling pressures and thereby diminish left ventricular work. In addition, TNG reduces resistance to coronary collateral flow and increases coronary flow to ischemic areas. Thus, TNG appears to have two effects that would contribute to an amelioration of ischemia. However, no information is available relating to the direct effects of nitroprusside on coronary arterial vessels. We therefore examined the ability of nitroprusside to modify the extent of experimentally induced acute myocardial ischemia, and compared the effects of this drug with those of nitroglycerin.

Five dogs with pre-existing multivessel coronary occlusive disease underwent acute balloon occlusion of the left anterior descending coronary artery. The extent of ischemic injury was determined by summating ST-segment elevation ($\Sigma$ST) from 7 subepicardial electrodes previously placed in the ischemic zone; measurements were made of heart rate, cardiac output, arterial pressure and left atrial pressure. Each animal was subjected to three occlusions in random order: 1) control (untreated), 2) following treatment with nitroprusside, 3) following treatment with nitroglycerin. Under control conditions, heart rate, arterial pressure, and left atrial pressure remained stable over the course of the 15 minute occlusion. $\Sigma$ST rose from preocclusion levels to a peak value at 10 minutes, and remained stable over the next five minutes of occlusion. Nitroprusside caused an average reduction in mean arterial pressure of 29% and an increase in heart rate of 29%. These changes were accompanied by an increase in $\Sigma$ST over control averaging 26% at 10 minutes and 34% at 15 minutes of occlusion ($p<0.05$). Nitroglycerin reduced arterial pressure 21% from control values and increased heart rate 29%. Despite

causing nearly identical hemodynamic effects as nitroprusside, however, nitroglycerin administration was accompanied by an average reduction in ΣST below control values of 13% at 10 minutes ($p<0.05$) and of 10% at 15 minutes. Thus in dogs with pre-existing multivessel coronary occlusive disease, none of whom had left ventricular failure, the extent of ischemic injury following experimental acute coronary occlusion is further aggravated by administration of nitroprusside, in contrast to the beneficial effect exerted by nitroglycerin. While perhaps not directly applicable to the clinical situation, these preliminary results suggest that the reduction in left ventricular work consequent to nitroprusside administration may not be accompanied by a reduction in ischemic injury; this finding emphasizes the point that therapy for acute myocardial infarction should be assessed in relation to its effects not only on hemodynamic function, but also on the magnitude of ischemic injury.

Keyword Descriptors: Nitroglycerin, Nitroprusside, Acute Myocardial Ischemia, Acute Coronary Occlusion, Vasodilators, Impedance Reduction, Dogs

Proposed Course of Project: Continuing

Honors and Awards: None

Publications: None

Project No. Z01 HL 01631-02 CB
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Nitroglycerin Therapy for Acute Myocardial Infarction in Man

Previous Serial Number: NHLI-120(c)

Principal Investigator: Jeffrey S. Borer, M.D.

Other Investigators: David R. Redwood, M.D.
Barrie Levitt, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: Cardiology Department, Flower and Fifth Avenue Hospitals, New York, N.Y.

Project Description: Previous investigations in dogs have demonstrated that nitroglycerin reduces ischemic injury and enhances ventricular electrical stability during acute coronary occlusion. These beneficial effects of nitroglycerin are potentiated by preventing drug-induced hypotension with an alpha-adrenergic agonist. Such combination therapy also has been found to reduce the incidence of spontaneous postocclusion ventricular fibrillation in dogs. The present study is designed to determine the efficacy of nitroglycerin, alone and with its hypotensive effects prevented with phenylephrine (an alpha agonist), in reducing the extent and severity of myocardial injury sustained during acute myocardial infarction in man. The efficacy of these interventions is being assessed by ST segment analysis of 35-lead precordial surface maps (experimental studies have demonstrated that changes in ST segment elevation reflect changes in myocardial ischemic injury).

Thus far, 12 patients with acute myocardial infarction have been studied. ST segment abnormalities have been reduced in each (17 to 60%) during therapy with sublingual nitroglycerin and phenylephrine. However, the optimal therapeutic regimen depended upon whether or not the patient was in left ventricular failure.

In the 7 patients without left ventricular failure, nitroglycerin administered alone caused a fall in blood pressure and a reflexly mediated increase in heart rate. These changes were not associated with a consistent decrease in the degree of ST segment elevation. In one patient it caused an excessive tachycardia, which led to an increase in the degree of ischemic injury. In contrast, when phenylephrine was added and infused at a rate sufficient to reverse the blood pressure lowering effects of nitroglycerin, significant improvement in the degree of myocardial ischemia

occurred in each patient.

In the 5 patients with left ventricular failure, nitroglycerin alone led to an average 15 mm fall in PA wedge pressure and a 26 mm Hg fall in mean arterial pressure. Despite the fall in arterial pressure, there was no reflex tachycardia. Moreover, in contrast to the patients without failure, TNG alone uniformly improved ST segment abnormalities. After abolition of the TNG-induced arterial pressure fall with phenylephrine, significant ST segment improvement was still present, but the benefit tended to be less marked than with nitroglycerin alone.

We conclude that ischemic injury occurring during acute myocardial infarction often, but not always, is reduced by nitroglycerin alone. When nitroglycerin and phenylephrine are given, ischemia is usually if not always diminished, with minimal risk of adverse effects. However, response of ischemia to therapy is not homogeneous, and cannot be predicted with certainty by monitoring hemodynamic variables alone. Therefore, at present it appears that while general guidelines, based on the presence or absence of failure, can be employed when initiating vasodilator therapy in the patient with an acute infarct, optimal therapy in the individual patient can be achieved only if a method such as precordial mapping is utilized for objective evaluation of ischemia.

Keyword Descriptors: Nitroglycerin, Ischemic Injury, Precordial Surface Maps, Phenylephrine, Acute Myocardial Infarction

Proposed Course of Project: Continuing

Honors and Awards: None

Publications: None

Project No. Z01 HL 01632-01 CB
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Effects of Vasodilators on Coronary Collateral Flow

Previous Serial Number: None

Principal Investigator: Norine L. Capurro, Ph.D.

Other Investigators: Kenneth M. Kent, M.D., Ph.D.
Stephen E. Epstein, M.D.

Cooperating Units: None

Project Description: Augmentation of coronary collateral flow is potentially beneficial to ischemic myocardium. Studies in the dog and man indicate that nitroglycerin enhances collateral flow. This study was designed to test pharmacological responsiveness of the coronary collateral circulation, and specifically to determine the effects of nitroglycerin and nitroprusside on coronary collateral flow. Dogs of either sex received general anesthesia, the heart was exposed through a left thoracotomy, and an ameroid constrictor was placed around the anterior descending (LAD) branch of the left coronary artery. The dogs were studied two to four weeks post-operatively. The dogs were anesthetized with sodium pentobarbital and the chest opened. A polyethylene cannula was inserted in the LAD distal to the ameroid. A Gregg cannula was inserted through the subclavian artery and secured in the left main coronary artery. Both the left main and the distal LAD were perfused with blood shunted from the dog's carotid artery. When inflow to the LAD cannula was clamped, peripheral coronary pressure (PCP) was measured and retrograde flow (RF) was collected from side arms of the LAD cannula. Systemic arterial pressure, left atrial pressure, ECG, and coronary flow were continuously monitored. Drugs were administered by constant intracoronary infusion (through the Gregg cannula) or by constant intravenous infusion. Since RF and PCP vary directly with perfusion pressure, arterial pressure was held constant at approximately 95 mm Hg, by manipulation of an inflatable cuff placed around the descending aorta. In 9 dogs with ameroid constrictors, control RF ranged from 11 to 90 ml/min (mean 35) and control PCP ranged from 54 to 98 mm Hg (mean 70). (In 4 normal dogs control RF ranged from 1.1 to 3.0 ml/min (mean 2.0) and control PCP ranged from 20 to 29 mm Hg (mean 24).) In six dogs, intracoronary (i.c.) administration of nitroglycerin, 0.3 to 100 µg/min, did not alter RF or PCP when systemic pressure was held constant at 95 mm Hg and with an average heart rate of 160 beats/min. In 2 dogs, heart rate was lowered by vagal stimulation to 60/min. In these dogs, nitroglycerin (i.c.) produced dose-dependent increases in RF but no change in PCP. Intravenous infusion of nitroglycerin, 10 to 300 µg/min, resulted in progressive increases in both RF and PCP at either rapid (2 dogs) or slow

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(3 dogs) heart rates. In 3 dogs, i.c. administration of nitroprusside, 0.3 to 100 µg/min did not alter RF or PCP. Further testing of the effects of nitroglycerin and nitroprusside, as well as those adrenergic drugs and changes of heart rate and vagal stimulation on RF and PCP are planned. These studies should help define the responsiveness of the coronary collateral circulation.

Keyword Descriptors: Coronary Collateral Flow, Retrograde Flow, Peripheral Coronary Pressure, Nitroglycerin, Nitroprusside

Proposed Course of Project: Continuing

Honors and Awards: None

Publications: None

Project No. Z01 HL 01634-02 CB
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

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Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Determinants of Ventricular Septal Motion

Previous Serial Number: NHLI 154(c)

Principal Investigator: Alan S. Pearlman, M.D.

Other Investigators: Chester E. Clark, M.D.
Joel Morganroth, M.D.
Walter L. Henry, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: None

Project Description: Echocardiographic studies of ventricular septal (VS) motion were performed in patients with a variety of cardiac disorders to define the determinants of septal motion. In 43 patients with right ventricular volume overload secondary to atrial septal defect, septal motion was frankly paradoxic in 35 (82%), flat in 4 (9%), and normal in 4 (9%). Pattern of septal motion was determined by the position of the VS relative to total cardiac diameter (distance from right ventricular epicardium to left ventricular (LV) epicardium at end-diastole). All patients with normal septal motion, and no patients with flat or paradoxic motion, had a septal position (defined as the distance from right ventricular epicardium to mid-septum/total cardiac diameter) at end-diastole $<.41$. When septal motion was measured (diastolic minus systolic septal position), the direction and magnitude of VS motion was linearly related to end-diastolic septal position ($r = .80$, $p<.01$). Septal motion correlated poorly with size of shunt ($r = .13$) and right ventricular pressure ($r = .18$). A similar relation between septal position and septal motion was evident in 1) 14 patients with other causes of right ventricular volume overload ($r = .82$), 2) 19 patients with LV volume overload ($r = .74$), 3) 10 patients with right ventricular pressure overload ($r = .93$), 4) 10 patients with LV pressure overload ($r = .80$), and 5) 28 normal subjects ($r = .82$). We conclude that the direction and magnitude of septal motion is determined by septal position relative to total cardiac transverse dimension. Thus, although paradoxic septal motion is usually seen in conditions causing right ventricular volume overload, it is not diagnostic of any particular hemodynamic burden. Rather, it is dependent upon the geometric position of the septum within the heart.

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Keyword Descriptors: Echocardiography, Atrial Septal Defect, Right Ventricular Volume Overload, Paradoxic Septal Motion, Congenital Heart Disease

Proposed Course of Project: Completed

Honors and Awards: None

Publications: None

Project No. Z01 HL 01636-01 CB
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Isolation and Characterization of Myosin From Patients With Asymmetric Septal Hypertrophy

Previous Serial Number: None

Principal Investigator: Barry J. Maron, M.D.

Other Investigators: Robert S. Adelstein, M.D.
Victor J. Ferrans, M.D., Ph.D.

Cooperating Units: Section of Pathology, NHLI

Project Description: Asymmetric septal hypertrophy (ASH) is a genetically transmitted disorder of cardiac muscle characterized by a disproportionately hypertrophied ventricular septum that contains numerous hypertrophied, bizarrely-shaped and disorganized cardiac muscle cells. These disorganized cardiac muscle cells are presumably the morphologic expression of the genetic defect in ASH. The present investigation was undertaken to characterize the biochemical and molecular characteristics of myosin from cardiac muscle cells of patients with obstructive ASH. Biochemical studies were performed on ventricular septal myocardium from 12 patients with obstructive ASH, left ventricular free wall myocardium from four patients with obstructive ASH and left ventricular free wall from four patients with normal hearts. Myosin was purified from preparations of actomyosin by gel filtration on columns of Sepharose 4B. Specific activity of myosin from the ventricular septum of patients with ASH ranged from 1.2 to 2.1 μmoles inorganic phosphate released per mg protein per minute (average, 1.6) and did not differ significantly from the specific activity of myosin from the left ventricular free wall of patients with obstructive ASH or of myosin from patients with normal hearts. Polyacrylamide-SDS gel electrophoresis of myosin from patients with obstructive ASH and patients with normal hearts demonstrated a heavy chain (molecular weight of 200,000 daltons) and two light chains (molecular weights of 25,000 and 20,000 daltons). Furthermore, characteristic bipolar aggregates of myosin molecules formed (at low ionic strength) in preparations of myosin from patients with ASH and from patients with normal hearts. Therefore, we have found no evidence to suggest that myosin from patients with obstructive ASH differs biochemically from that of patients with normal hearts or that myosin from the ventricular septum of patients with obstructive ASH differs from myosin from the left ventricular free wall of the same patients.

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Keyword Descriptors: Myosin, Idiopathic Hypertrophic Subaortic Stenosis, Hypertrophic Cardiomyopathy, Polyacrylamide-SDS Gel Electrophoresis, Ultrastructure; Myosin ATPase activity

Proposed Course of Project: Completed

Honors and Awards: None

Publications: None

Project No. Z01 HL 01637-01 CB
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Indices of Reversibility in Heart Failure

Previous Serial Number: None

Principal Investigator: Barry J. Maron, M.D.

Other Investigators: Kenneth M. Kent, M.D.
Bruce Reitz, M.D.
Jack Copeland, M.D.
James Scherer, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: Clinic of Surgery, NHLI and Radiology Department, NIH

Project Description: To determine factors responsible for irreversible cardiac failure, hemodynamic studies were performed in 11 dogs with circulatory overload (secondary to aorto-caval shunt) and again late after reversal of the shunt. Clinical and hemodynamic evidence of LV failure appeared an average of 55 days after exposure to volume overload:

| | LVEDP | LVEDV | EF | MCF |
|---|---|---|---|---|
| Control dogs | 3±1 | 33±7 | .78±.14 | .76±.12 |
| Shunted dogs | 24±11* | 102±42* | .49±.2* | .31±.06* |

*=p<.01. (LVEDP=LV end-diastolic pressure; LVEDV=LV end-diastolic volume; EF=ejection fraction; MCF=mean rate circumferential fiber shortening). Late after shunt closure (avg 73 days) EF (.48±.17), MCF (.46±.08) had not changed significantly from preclosure values. LVEDP (10±6 mm Hg), LVEDV (64±18 ml) decreased significantly compared to preclosure ($p<.02$) but were elevated over controls ($p<.01$). LVEDV late after shunt closure was directly related to the magnitude of the initial shunt ($p<.05$). There was no relation between LVEDP, LVEDV, EF, MCF, dp/dt measured prior to and late after shunt closure. Thus, in dogs with volume overload, magnitude of shunt is a reliable predictor of residual LV dilatation. However, usually employed hemodynamic indices of cardiac function are of little value in predicting potential for reversibility of cardiac failure.

Keyword Descriptors: Heart Failure, Hemodynamics, Arteriovenous Shunt, Ventricular Volume Overload

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Proposed Course of Project: Continuing

Honors and Awards: None

Publications: None

Project No. Z01 HL 01639-03 CB
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Echocardiographic Characteristics of Infiltrative Cardiomyopathy

Previous Serial Number: NHLI-6(c)

Principal Investigator: Jeffrey S. Borer, M.D.

Other Investigators: Walter L. Henry, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: None

Project Description: Echocardiography (ECHO) was used to evaluate the cardiac status of adults with systemic diseases (amyloidosis, 4 pts; hemosiderosis, 5 pts; mucopolysaccharidosis, 4 pts; idiopathic hypereosinophilia, 10 pts) known to be associated with infiltrative cardiomyopathy. Though 8 (35%) pts had no other clinical or non-invasive laboratory evidence of cardiac disease, all had ECHO abnormalities consistent with infiltrative disease. LV mass was elevated (>275 gm) in 23 (88%), (mean 385 gm, nl 211, $p < .01$); LV free wall and septum were symmetrically thickened ( >11 mm) in 23 pts (mean 14.5 mm, nl 9.8, $p < .01$); LV internal dimension was abnormal in 7 pts. Mitral valve closure slope was reduced (<125 mm/sec) in 20 pts (mean 87 mm/sec, nl 147, $p < .01$), consistent with diminished LV compliance. However, systolic function was well maintained (ejection fraction mean 7%, nl 71%, n.s.). These ECHO findings were commonly associated with each disease studied. We conclude that in adults with diseases known to be associated with infiltrative cardiomyopathy 1) ECHO is a sensitive technique for detecting abnormalities in patients with clinically inapparent cardiac disease, 2) ECHO studies frequently demonstrate symmetrically increased LV wall thickness and LV mass and 3) systolic function is preserved while diastolic compliance is reduced.

Keyword Descriptors: Echocardiography, Cardiomyopathy, Amyloidosis, Hemosiderosis, Hypereosinophilia, Mucopolysaccharidosis.

Proposed Course of Project: Continuing

Honors and Awards: None

Publications: None

Project No. Z01 HL 01641-01 CB
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Growth of Aortic Smooth Muscle Cells In Vitro

Previous Serial Number: None

Principal Investigator: Chester E. Clark, M.D.

Other Investigators: Stephen E. Epstein, M.D.

Cooperating Units: None

Project Description: There is evidence that the intimal plaque in atherosclerosis is initially derived from smooth muscle cells found in the media. Several investigators have cultivated these cells, obtained from animals, and have shown that their growth rate can be modified by manipulating their environment, most notably the lipid content of the culture medium. We have attempted to grow cells from the proximal aorta of patients undergoing cardiac surgery, in an effort to understand some of the factors that control growth and reproduction of these cells. Using a variety of culture media, we have successfully grown human aortic cells, and are preparting to manipulate their growth.

Keyword Descriptors: Atherosclerosis, Aortic Smooth Muscle Cells, Lipids, Tissue Culture

Proposed Course of Project: Continuing

Honors and Awards: None

Publications: None

Project No. Z01 HL 01642-01 CB
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Physical Factors Determining Cardiac Motion

Previous Serial Number: None

Principal Investigator: Chester E. Clark, M.D.

Other Investigators: Alan S. Pearlman, M.D.
Walter L. Henry, M.D.

Cooperating Units: None

Project Description: There have been numerous studies in both animals and humans providing descriptive data relative to the motion of cardiac structures. Nevertheless, there have been few efforts to provide a systematic analysis based on physical principles. We studied 18 patients with two-dimensional echocardiography: eight normals, six patients with right ventricular volume overload, two with left ventricular volume overload, two with right ventricular pressure overload, one with left ventricular pressure andright ventricular volume overload, and one with an enlarged right ventricle but no volume or pressure load. The data allowed us to test and validate a hypothesis that overall cardiac movement results from recoil consequent to ejection of blood into the great vessels and an additional internal movement of structures during systole toward the center of mass of the heart. We hypothesized and subsequently confirmed that the motion of any given point within the heart can be found by adding the recoil force to the movement about the center of mass. This hypothesis accounts for the observed motion of cardiac structures, and in particular explains the motion of the ventricular septum that is seen in a variety of cardiac disorders.

Keyword Descriptors: Cardiac Motion, Ventricular Septum, Center of Mass

Proposed Course of Project: Completed

Honors and Awards: None

Publications: None

Project No. Z01 HL 01643-02 CB
1. Cardiology
2. Clinical Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Growth of Cells in Tissue Culture From Patients with ASH

Previous Serial Number: NHLI-158(c)

Principal Investigator: Chester E. Clark, M.D.

Other Investigators: None

Cooperating Units: None

Project Description: We have shown that ASH is a genetically determined disease that is transmitted as an autosomal dominant trait. In an effort to understand the basic cellular defect, we have attempted to cultivate cells obtained at operation from the ventricular septum of patients with ASH. Initial efforts involving enzymatic dissociation of the cells failed. Subsequently, we used an explant technique that yielded a very slow outgrowth of cells. Morphologically, these cells appear to be large, bizarre fibroblasts. We have not positively identified any cardiac muscle cells.

At the present time, we are culturing rat skeletal-muscle tumor cells in an effort to select mutants that are resistant to metabolic poisons. Both 8-azaguanine and 6-thioguanine are being used to select cells that lack the enzyme 5-PRPP. Our intention is to hybridize these mutant skeletal muscle cells with cardiac cells from patients with ASH and study the process of myotube formation as it is affected by the presence of abnormal genetic material from the ASH cells. In this manner, we hope to develop some insight into the nature of the genetic defect in ASH.

Keyword Descriptors: ASH, Skeletal Muscle Cells, Hybridization

Proposed Course of Project: Continuing

Honors and Awards: None

Publications: None

Project No. Z01 HL 01607-02 CB
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Dynamic EKG-gated Scintiangiography

Previous Serial Number: NHLI-145(c)

Principal Investigators: William R. Brody, M.D.
Michael V. Green, Ph.D.

Other Investigators: Alan S. Pearlman, M.D.
Harold G. Ostrow, B.S.E.E.
Margaret Douglas, B.A.
James J. Bailey, M.D.
Gerald S. Johnston, M.D.
Samuel B. Itscoitz, M.D.
David R. Redwood, M.D.

Cooperating Units: Laboratory of Nuclear Medicine; Division of Computer Research and Technology

Project Description: Using intravenous $^{99m}Tc$ human albumin, an EKG-gated, computer-based, scintigraphic imaging procedure that produces a sequence of consecutive 10-millisecond images to represent a single, complete, average cardiac cycle was evaluated. Repetitive projection of this image sequence as a scintigraphic cineangiogram permits visualization of the dynamic behavior of the entire heart and associated vasculature during both systole and diastole and can reveal defects such as myocardial akinesis.

The picture sequence was also investigated quantitatively to directly yield ejection duration and to estimate ejection fraction. Relative (and potentially absolute) measures of instantaneous ventricular volume and maximum instantaneous flow can also be made.

Limitations to the quantitative aspects of the procedure include those imposed by variable R-R interval lengths, uncertainties in the calculation of ventricular background and various physical and subject-detector geometry considerations.

The procedure offers several advantages over other techniques for assessing ventricular function. It is non-invasive, repeatable, may be performed by technical personnel and can yield information not readily obtained by other methods. A series of 30 patients undergoing routine cardiac catheterization has been studied by this scintigraphic technique.

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Preliminary results suggest an excellent correlation between parameters of ventricular function measured by the two methods.

Keyword Descriptors: Intravenous $^{99m}$Tc Human Albumin, Cardiac Cycle, Ejection Fraction, Ventricular Volume

Proposed Course of Project: Continuing

Honors and Awards: None

Publications: None

Project No. Z01 HL 01609-02 CB
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Scintigraphy in the Assessment of Coronary Artery Disease

Previous Serial Number: NHLI-144(c)

Principal Investigators: David R. Redwood, M.D.

Other Investigators: Harry Agress,Jr., M.D.
William R. Brody, M.D.
Michael V. Green, Ph.D.
James J. Bailey, M.D.
Alan S. Pearlman, M.D.
Samuel B. Itscoitz, M.D.
Gerald S. Johnston, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: Laboratory of Nuclear Medicine, NIH

Project Description: The precise pathophysiologic significance of subcritical coronary narrowing is unknown. It is generally accepted that coronary lesions producing 50% stenosis or less are of no functional significance; hence, patients with such lesions are not considered candidates for bypass surgery. Recent studies using a dual isotope technique to assess relative myocardial perfusion, however, suggest that inadequate perfusion after a vasodilatory stimulus can result from coronary lesions causing as little as 50% luminal narrowing. These findings pose important therapeutic questions. We therefore are evaluating the relative significance of coronary stenotic lesions of varying severity by studying adequacy of regional myocardial perfusion at rest and at the time of pacing induced angina by the use of intracoronary injections of labeled macro-aggregated albumin (MAA) and human albumin microspheres (HAM).

Patients investigated are those who undergo diagnostic cardiac catheterization including left ventricular angiography and selective coronary arteriography for medical indications unrelated to this study. The patient's stress threshold is then determined by successively increasing heart rate by right atrial pacing until 85% of maximum predicted heart rate or angina occurs. Pacing is then discontinued allowing rapid resolution of angina and a 5-10 min stabilization will ensue. For the resting scintigram, either $^{99m}$Tc-labeled HAM (consisting of 1-1.5 mCi, 10,000 to 30,000 particles with diameter 10-40 μ suspended in 5cc saline) or $^{131}$I labeled MAA (100 μCi, 200,000-400,000 particles with diameter of 10-80 μ suspended in 5cc saline)is injected into each coronary artery. Pacing is then initiated until the stress threshold is attained. At this point, $^{99m}$Tc-labeled HAM or $^{131}$I labeled MAA is injected into the left coronary artery after which pacing is discontinued.

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Following a five-minute rest period, pacing is repeated as before and $^{131}$I-MAA injected into the right coronary artery at the point of maximum stress. Myocardial images representing resting and stress scintigrams are then obtained and compared to assess stress induced changes in regional perfusion. Finally, in order to evaluate the functional significance of different degrees of narrowing, adequacy of regional perfusion during pacing is correlated with degree of coronary stenosis as judged from coronary angiograms. Preliminary results suggest an excellent correlation between patterns of perfusion abnormality and the degree and extent of disease in the coronary arteries. In addition, there is a correlation between these perfusion abnormalities and the segmental ventricular wall motion abnormalities as seen on ventricular cine-angiography.

Keyword Descriptors: Dual isotope technique, Regional myocardial perfusion, Cardiac catheterization, Selective coronary arteriography, Pacing.

Proposed Course of Project: Continuing

Honors and Awards: None

Publications: None

Project No. Z01 HL 01610-02 CB
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Stress Myocardial Imaging in the Evaluation of Cardiac Disease

Previous Serial Number: NHLI-142(c)

Principal Investigator: Richard W. Myers, M.D.

Other Investigators: Robert E. Goldstein, M.D.
Gerald S. Johnston, M.D.
Stephen E. Epstein, M.D.
David R. Redwood, M.D.

Cooperating Units: Laboratory of Nuclear Medicine, NIH

Project Description: The feasibility of demonstrating localized ischemia as "cold areas" in a $^{43}K$ myocardial scan obtained after graded exercise has recently been reported. The underlying mechanism of this finding probably involved increased potassium turnover in ischemic myocardium in addition to decreased potassium delivery by diseased vessels. If systematic evaluation shows acceptable diagnostic accuracy, a wide application to the assessment of coronary artery disease may be expected, as this technique would allow screening of large numbers of susceptible patients as well as provide useful information in evaluating patients for more definitive techniques such as coronary angiography.

In applying stress myocardial imaging, however, consideration must be given to other cardiac diseases that may involve regional myocardial ischemia and hence give false positive results. Asymmetric septal hypertrophy may be such a condition. Its pathologic picture is that of non-uniform left ventricular hypertrophy. The frequent clinical findings of exertional chest pain and EKG evidence of ischemia or previous myocardial infarction further complicate the differentiation of asymmetric septal hypertrophy from coronary artery disease.

The purpose of this study, then, was first to evaluate the diagnostic reliability of stress myocardial scintigraphy in patients with coronary artery disease, and second, to describe localized abnormalities in patients with asymmetric septal hypertrophy.

Each patient underwent myocardial imaging on two occasions. At least four days (four half-lives of $^{43}K$) prior to exercise study, a baseline study was performed after intravenous injection of 0.5 to 1.0 mCi of $^{43}KCl$. The patient then performed a standard graded exercise protocol. At the end



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point of the stress test (angina, fatigue, dyspnea, or reaching 85% of maximum predicted heart rate), 0.5 to 1.0 mCi of $^{43}$KCl was injected intravenously. The patient was transported to the Nuclear Medicine Laboratory and myocardial imaging performed as in the baseline study.

A comparison of results of the independent evaluation of resting and stress myocardial imaging to other diagnostic studies allowed determination of 1) the accuracy of detection of ischemia secondary to coronary artery disease, and 2) the assessment of the character and occurrence of defects in asymmetric septal hypertrophy.

The presence of myocardial defects in patients with coronary artery disease appeared relatively specific when compared to normal subjects (positive in 7 of 10 patients with coronary artery disease; 0 of 5 normal patients). However, 3 of 7 patients with asymmetric septal hypertrophy showed similar defects. The technique also was relatively insensitive in that 3 of 10 patients with coronary artery disease had negative studies.

Keyword Descriptors: $^{43}$K Myocardial Scan, Coronary Artery Disease, Asymmetric Septal Hypertrophy, Graded Exercise

Proposed Course of Project: Completed

Honors and Awards: None

Publications: None

Project No. Z01 HL 01611-02 CB
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Pre- and Postoperative Exercise Performance in Patients with ASH

Previous Serial Number: NHLI-163

Principal Investigators: Robert E. Goldstein, M.D.
David R. Redwood, M.D.

Other Investigators: Samuel B. Itscoitz, M.D.
Andrew G. Morrow, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: Clinic of Surgery, NHLI

Project Description: Operative relief of left ventricular outflow tract obstruction due to asymmetric septal hypertrophy (ASH) frequently produces subjective improvement in symptoms. However, little objective data have been obtained concerning the effects of this operation on exercise performance. Although the ability of left ventricular myotomy and myectomy to eliminate the left ventricular outflow tract gradient is well established, the extent of compromise in exercise performance due to persisting myopathy remains uncertain. We have therefore assessed the ability of patients to perform treadmill exercise by measuring exercise endurance and maximal oxygen consumption before and after left ventricular myotomy and myectomy. To gain insight into the effects of operation on the hemodynamic response to exercise, patients have performed upright exercise with simultaneous measurement of cardiac output and pulmonary arterial and systemic arterial pressures and oxygen contents. To date, complete preoperative and postoperative exercise evaluation has been performed in ten individuals. Preliminary data indicate that substantial improvement in exercise capacity is observed after operation in some but not all individuals. Insufficient data are available at present to relate the degree of benefit to clinical or laboratory findings.

Keyword Descriptors: Asymmetric Septal Hypertrophy, Myotomy and Myectomy, Treadmill Exercise, Maximal Oxygen Consumption, Cardiac Output

Proposed Course of Project: Continuing

Honors and Awards: None

Publications: None

Project No. Z01 HL 01612-02 CB
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Left Ventricular Function Using Roentgen Videodensitometry

Previous Serial Number: NHLI-117(c)

Principal Investigator: David R. Redwood, M.D.

Other Investigators: Leonard E. Grauer, M.D.
William H. Schuette, B.E.E.
Willard C. Whitehouse, B.S.
Samuel B. Itscoitz, M.D.

Cooperating Units: Biomedical Engineering and Instrumentation Branch
Division of Research Services, Television Engineering Section, Clinical Center

Project Description: Left ventricular ejection fraction, the ratio between stroke volume and end-diastolic volume, is widely used as an index of ventricular function in patients with myocardial and valvular heart disease. However, as conventionally determined, the calculation of end-diastolic and end-systolic ventricular volumes involves planimetry of appropriate frames of the left ventricular cineangiogram, which is a time consuming and cumbersome undertaking. Because of the difficulties of this method, an automated technique has been developed that allows for the direct measurement of ejection fraction.

This technique determines the rate of wash-out of roentgen dense contrast material from the left ventricle during cineangiography. A densitometer placed over the image of the left ventricle continuously measures the changes in contrast density during the cine run. The degree to which the contrast is cleared from the left ventricle with each systole is a function of the ejection fraction and can be determined by finding the number of cardiac cycles (N) necessary to wash out 50% of the contrast. The ejection is then equal to $1-E^{-0.693/N}$ (the equation for an exponential curve). Results in 29 patients using this method show an excellent correlation with data obtained by planimetry techniques (r=0.81).

Keyword Descriptors: Ejection fraction, Ventricular volumes.

Proposed Course of Project: Completed

Honors and Awards: None

Project No. Z01 HL 01612-02 CB
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Maryland

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Publications: Measurement of Ejection Fraction in Man by Videodensitometry. Schuette, W.H., Grauer, L.E., Whitehouse, W.C., Itscoitz, S.B., Redwood, D.R. Catheterization and Cardiovascular Diagnosis, in Press.

Project No. Z01 HL 01616-02 CB
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Effect of TNG during exercise in Valvular Heart Disease

Previous Serial Number: NHLI-119(c)

Principal Investigator: Jeffrey S. Borer, M.D.

Other Investigators: David R. Redwood, M.D.
Samuel B. Itscoitz, M.D.
Robert E. Goldstein, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: None

Project Description: Nitroglycerin reduces pulmonary arterial and left atrial pressures and pulmonary vascular resistance at rest in patients with mitral stenosis. We have observed similar changes in patients with aortic insufficiency and mitral insufficiency. Changes in cardiac output during such studies have been variable. To assess the potential clinical utility of nitroglycerin in patients with valvular heart disease we are determining the effect of nitroglycerin 1) on treadmill exercise capacity and 2) on the pulmonary artery pressure and cardiac output response to exercise. Thus far, 9 patients have been studied, most with mild mitral and aortic valve lesions. Of these, 8 have had technically adequate evaluations of exercise tolerance; all have shown increases in exercise tolerance during nitroglycerin therapy as compared with placebo therapy; tolerance had increased an average of 20% after 0.5 mg sublingual nitroglycerin; four patients have undergone technically adequate evaluation of pulmonary artery pressure and cardiac output response to exercise. In all patients, pulmonary artery pressure has been lower and cardiac output higher at maximal exercise during nitroglycerin therapy than during placebo therapy.

Our results suggest that vasodilator therapy may be a useful adjunct in the pharmacologic management of patients with valvular heart disease by reducing exertional symptoms and increasing exercise tolerance.

Key Word Descriptors: Treadmill, Cardiac Output, Pulmonary Artery Pressure

Proposed Course of Project: Continuing

Honors and Awards: None

Publications: None

Project No. Z01 HL 01633-01 CB
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Persistent Paradoxic Septal Motion Following ASD Closure

Previous Serial Number: None

Principal Investigator: Alan S. Pearlman, M.D.

Other Investigators: Walter L. Henry, M.D.
Chester E. Clark, M.D.
Samuel B. Itscoitz, M.D.
David R. Redwood, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: None

Project Description: Previous echocardiographic studies have shown persistent paradoxic ventricular septal motion in some patients following atrial septal defect (ASD) repair. While the possibility of residual shunt has been suggested to explain this observation, the mechanism underlying persistent abnormal septal motion remains unclear. Recently, we noted that paradoxic ventricular septal motion is dependent upon the relative degree of posterior displacement of the septum within the total ventricular mass at end-diastole. We therefore sought to determine whether such a mechanism might explain persistent postoperative paradoxic motion. Nine patients with ASD were studied: all had preoperative and postoperative right heart catheterization and echocardiographic studies. Preoperatively, 5 patients had paradoxic, 3 flat, and 1 normal septal motion. Pulmonary:systemic flow ratios ranged from 1.6-5.6:1, and right ventricular diastolic diameter index (RVDDI) ranged from 1.6-3.3 (normal 0.6-1.4). Postoperatively, 2 patients had paradoxic, 4 flat and 3 normal septal motion. No patient had a residual shunt. The 3 patients with normal motion postoperatively all had return of RVDDI to normal. One patient with flat motion preoperatively developed marked pulmonary hypertension, an increased RVDDI, and paradoxic motion postoperatively; 5 patients had only a small decrease in RVDDI and abnormal motion persisted postoperatively. There was a significant relation between the intracardiac position of the septum at end-diastole and the direction and magnitude of septal motion during systole in both preoperative ($r = 0.80$) and postoperative ($r = 0.66$) patients. We conclude that persistent paradoxic motion after ASD repair reflects incomplete resolution of right ventricular dilatation and not necessarily residual shunt.

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Key Word Descriptors: Echocardiography, Atrial Septal Defect, Right Ventricular Dilatation, Paradoxic Septal Motion, Congenital Heart Disease

Proposed Course of Project: Continuing

Honors and Awards: None

Publications: None

Project No. Z01 HL 01635-01 CB
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Scintigraphic Detection of Asymmetric Septal Hypertrophy

Previous Serial Number: None

Principal Investigator: Alan S. Pearlman, M.D.

Other Investigators: Michael V. Green, Ph.D.
Harry Agress, Jr., M.D.
William R. Brody, M.D., Ph.D.
David R. Redwood, M.D.
Gerald H. Johnston, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: Laboratory of Nuclear Medicine, Division of Computer Research and Technology

Project Description: Recently, we described the use of ECG-gated scintigraphic angiocardiography for the quantitative analysis of left ventricular (LV) function. During these studies, it became apparent that his technique permitted visualization of the configuration of the ventricular septum. Since abnormalities of LV function and septal configuration characterize patients with asymmetric septal hypertrophy (ASH), we investigated the ability of scintigraphy to detect abnormalities diagnostic of ASH. Based on echocardiographic studies, patients were assigned to one of three groups: ASH (7 pts), concentric thickening (7 pts), and normal septum (7 pts). Following peripheral intravenous injection of 10 mCi of $Tc^{99m}$-human serum albumin, scintigraphic scans were obtained in the left anterior oblique position. With the aid of computer techniques, an "average" cardiac cycle was generated for each patient and from it an LV volume curve. The end-diastolic image, volume curve, and derived ejection fraction (EF) were examined. In 6 (86%) of the patients with ASH, the right and left septal surfaces wery not parallel at end-diastole; in 4 (57%) of these patients, there was a protrusion of the septum high in the LV outflow tract. Five patients (71%) with ASH had an unusually high EF (.69 or greater); 5 (71%) had an increased atrial component of the LV volume curve, suggesting decreased LV compliance. In contrast to the results of the patients with ASH, images from all 14 patients with either a normal or concentrically thickened septum demonstrated parallel right and left septal surfaces, and EF in each patient was less than .69. Three (21%) of these patients had an increased atrial component of the LV volume curve. We conclude that scintigraphic scanning is a safe, non-invasive method for detecting the lack of parallelism of septal surfaces, high EF, and decreased LV compliance characteristic of ASH. While less

sensitive and more complicated than echocardiography, scintigraphy may be useful in the noninvasive screening of patients suspected of having ASH when echocardiography is technically unsatisfactory. In addition, since LV geometry is usually distorted in patients with ASH, accurate assessment of EF by angiography or echocardiography probably is unreliable. The technique we have described, however, allows measurement of EF without recourse to any assymptions about LV geometry and thus may be helpful in defining disease progression, and in assessing the effects of therapeutic interventions.

Keyword Descriptors: Radioisotope Imaging, Asymmetric Septal Hypertrophy, Compliance Cardiomyopathy, Noninvasive Techniques, IHSS

Proposed Course of Project: Continuing

Honors and Awards: None

Publications: None.

Project No. Z01 HL 01638-01 CB
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Interventricular Septum in ASH

Previous Serial Number: None

Principal Investigator: David R. Redwood, M.D.

Other Investigators: William C. Roberts, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: Section on Pathology, Division of Intramural Research.

Project Description: A previous angiographic study from our laboratory suggested that the configuration of the ventricular septum in patients with asymmetric septal hypertrophy (ASH) and left ventricular outflow obstruction (obstructive ASH) was different from that of patients without obstruction (nonobstructive ASH). To confirm and extend these findings, a necropsy study of the septum has been carried out in 18 patients with documented ASH. The septum was cut parallel to the long axis of the heart in a plane lying between the right coronary and non-coronary aortic leaflets. In nonobstructive ASH, the membranous septum lay at the apex of a triangular muscular septum, the sides of which diverged inferiorly to the right and left. In contrast, the membranous septum in obstructive ASH joined the muscular septum in a line continuous with the right endocardial border of the septum, while the left septal border was convex and encroached into the left ventricular outflow tract. Thus, the septum achieved its maximal width more cephalad and the ratio of upper to lower septal width was greater ($1.1 \pm .02$ vs. $.85 \pm .02$, $p < .005$) in obstructive than in nonobstructive ASH. There was no difference between obstructive and nonobstructive ASH in septal length nor in septal width at the septal equator. This study is consistent with the results of angiographic studies and suggests that differences in septal width and contour may be causally related to the genesis of obstruction to left ventricular outflow in ASH.

Keyword Descriptors: Asymmetric Septal Hypertrophy, Ventricular Septum, Left Ventricular Outflow Obstruction

Proposed Course of Project: Completed

Honors and Awards: None

Publications: None

Project No. Z01 HL 01640-02 CB
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Limitations of the Electrocardiographic Response to Exercise in Predicting Coronary Artery Disease

Previous Serial Number: NHLI-118(c)

Principal Investigator: Jeffrey S. Borer, M.D.

Other Investigators: John F. Brensike, M.D.
David R. Redwood, M.D.
Samuel B. Itscoitz, M.D.
Eugene R. Passamani, M.D.
Neil J. Stone, M.D.
John M. Richardson, M.D.
Robert I. Levy, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: Molecular Disease Branch, NHLI
Division of Heart and Vascular Disease, NHLI

Project Description: The electrocardiographic (ECG) response to graded bicycle exercise (to 85% of predicted maximal heart rate) was evaluated prior to coronary angiography in 89 patients, aged 21 to 55 years, with type II hyperlipoproteinemia. Patients were studied if they had a) a history of myocardial infarction and/or typical angina (43 patients), b) "atypical angina" (16 patients), or c) a positive ECG response during or shortly after exercise, but were otherwise without evidence of cardiac disease (30 patients) Of the 43 Group A patients, 39 had $\geq$50% stenosis; however, 26 (67%) of these 39 had negative ECG tests, including 9 of 17 patients with $\geq$50% stenosis in each of 3 vessels. Of the 16 Group B patients, 5 had $\geq$50% stenosis, 3 with positive ECG tests; 1 patient had a positive test but normal arteriogram. Of the 30 Group C patients, 11 (37%) had $\geq$50% stenosis; however, 9 (30%) had minor stenoses ($\leq$50%) and 10 (33%) had entirely normal coronary arteries. Therefore, exercise electrocardiography results in a high frequency of both false negative responses (in patients with clinically suspected coronary disease) and false positive responses (in asymptomatic patients). We conclude that while exercise electrocardiography may be of value in epidemiologic studies, its applicability as a diagnostic tool in the individual patient has marked limitations.

Keyword Descriptors: ECG, Exercise, Type II Hyperlipoproteinemia, Angina, False Negative Responses, False Positive Responses



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Proposed Course of Project: Completed

Honors and Awards: None

Publications: Manuscript submitted to the NEW ENGLAND JOURNAL OF MEDICINE

Project No. Z01 HL 01644-04 CB
1. Cardiology
2. Molecular Cardiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Phosphorylation of Myosin from Muscle and Non-muscle Sources

Previous Serial Number: NHLI-18

Principal Investigator: Robert S. Adelstein, M.D.
James Daniel, Ph.D.
Mary Anne Conti, B.S.

Other Investigators: William Anderson,Jr., B.A.

Cooperating Units: None

Project Description: A protein kinase with a molecular weight of approximately 80,000 daltons has been isolated from human platelets. This kinase catalyzes the phosphorylation of the 20,000 dalton light chain of platelet myosin as well as the 20,000 dalton light chain of mouse fibroblast, chicken gizzard and a myosin isolated from a muscle tumor. Normal human skeletal and rabbit skeletal myosin are not phosphorylated by the enzyme but inhibit the enzymes ability to phosphorylate platelet myosin light chain.

The effect of phosphorylation on platelet myosin is to increase (5-8 fold) the actin-activated ATPase activity measured in the presence of $Mg^{2+}$ at low ionic strength. It has no effect on the platelet myosin ATPase activity measured in the presence of $Ca^{++}$ and $K^{+}$ - EDTA at high ionic strength. Dephosphorylation of previously phosphorylated platelet myosin resulted in a decrease in the actin-activated ATPase activity without alteration of the $Ca^{++}$and $K^{+}$ - EDTA activated myosin ATPase activity.

Keyword Descriptors: Platelet myosin; non-muscle myosin phosphorylation; kinase; actin-activation.

Proposed Course of Project: The mechanism by which phosphorylation of platelet myosin results in actin-activation will be studied. The possibility that phosphorylation leads to actin-activation in other non-muscle cells such as fibroblasts as well as smooth muscle myosin, will be investigated.

Evidence that phosphorylation of platelet myosin plays a role in the platelet release reaction will be studied by looking for increased incorporation of $^{32}P_i$ into platelet light chain following treatment of human platelets with thrombin or ADP. Recent results in this laboratory by Dr. Daniel suggest that this may be the case.

Since the effect of phosphorylation appears to be reversible, the phosphatase



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responsible for dephosphorylation will be investigated and isolated in human blood platelets.

Honors and Awards: None

Publications: Articles published:

1) Adelstein, R.S., Conti, M.A., Daniel, J.L. and Anderson,Jr.,W. The Interaction of Platelet Actin, Myosin and Myosin Light Chain Kinase. (1975), Ciba Foundation Symposium on the Biochemistry and Pharmacology of Blood Platelets, New Series (In press).

2) Adelstein, R.S., Daniel, J.L., Conti, M.A. and Anderson,Jr.,W. Platelet Myosin Phosphorylation: Studies on the Kinase Substrate and Effect of Phosphorylation. Proceedings of the 9th FEBS Meeting (1974, in press).

Project No. Z01 HL 01645-02 CB
1. Cardiology
2. Molecular Cardiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Contractile Proteins from Adult, Embryonic and Malignant Cells

Previous Serial Number: NHLI-164

Principal Investigator: Robert S. Adelstein, M.D.
Mary Anne Conti, B.S.

Other Investigators: William Anderson, Jr., B.A.
Donald Henson, M.D.
John Ziegler, M.D.
Marshall Elzinga, Ph.D.
Guilio Cantoni, Ph.D.

Cooperating Units: Biomedical Research Institute, Boston
NCI
NICHHD
NIMH

Project Description: Non-muscle myosin, characterized by a unique set of light chains compared to skeletal muscle and cardiac myosin is thought to play a role in cell division, cell secretion, cell motility and specialized cell function such as clot retraction in platelets and phagocytosis in white cells. In order to elucidate such roles we have embarked on a study of myosin in a) myoblasts before and after fusion, b) muscle tumors such as rhabdomyosarcoma, as well as non-muscle tumors.

The rationale for these experiments is the concept that myoblasts prior to fusion and muscle tumor cells having undergone dedifferentiation, would both contain a cytoplasmic type myosin, similar to that found in fibroblasts and platelets. Evidence for such a myosin would be the presence of the unusual light chains (20,000 and 15,000 daltons) associated with "non-muscle" myosin and the ability of the 20,000 dalton light chain to be phosphorylated by the kinase from platelets which does not phosphorylate skeletal muscle or cardiac myosin. Initial experiments in this laboratory with myoblasts prior to fusion and rhabdomyosarcoma cells removed as operative specimens and grown in vitro have supported the hypothesis. The molecular weight of the light chains of myoblast myosin prior to fusion was found to be 20,000 and 15,000 daltons, and after fusion to be 25,000, 20,000 and 18,500 (similar experiments with comparable findings were carried out in Dr. Howard Holtzer's laboratory at the University of Pennsylvania in Philadelphia).

Rhabdomyosarcoma cells from operative specimens were found to contain a mixture of light chains including the 20,000 and 15,000 dalton light chains.

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Individual Project Report
July 1, 1974 through June 30, 1975

Dr. Marshall Elzinga in Boston has been sequencing two actins prepared in this laboratory: a) actin prepared from human platelets and b) actin prepared from human hearts (autopsy specimens). The purpose of this work is to uncover any differences between muscle and non-muscle actin. Two cyanogen bromide peptides of human platelet actin comprising 20 residues have been found to have the exact sequence as rabbit skeletal muscle myosin. One peptide of 9 residues has a single amino acid substitution (threonine for valine).

Keyword Descriptors: Myosin light chains; cell proliferation; cell division; cell motility; atherosclerosis; rhabdomyosarcoma.

Proposed Course of Project: Myosin and actin from a number of malignant and non-malignant but actively proliferating cells (muscle and non-muscle) will be compared with regard to structure, function and antigenic determinants with myosin from cells not undergoing active cell division. Of particular interest (in addition to the cells mentioned above) will be the isolation and characterization of myosin from the media of aortic cells undergoing proliferation which is thought to be involved in the genesis of atherosclerosis.

Actin and myosin will be characterized from cells that have been found to be abnormal in cell motility in an effort to uncover the cause of this abnormality.

Honors and Awards: None

Publications: Ostlund, R.E., Pastan, I. and Adelstein, R.S.: Myosin in cultured fibroblasts. J. of Biol. Chem 249: 3903-3907, 1974.

Kuehl, W.M., Conti, M.A. and Adelstein, R.S.: Structural studies on rabbit skeletal muscle actin: Ordering of the peptides produced by cleavage with cyanogen bromide. J. of Biol. Chem. 250, 1975 (in press).

ANNUAL REPORT OF THE
HYPERTENSION-ENDOCRINE BRANCH
NATIONAL HEART AND LUNG INSTITUTE
July 1, 1974 through June 30, 1975

The activities of the Hypertension-Endocrine Branch have been primarily devoted to studies of the chemistry, physiology and clinical applications of systems known or thought to exert controlling influence on blood pressure; these include especially 1) the autonomic nervous system, 2) the renin-angiotensin system 3) the prostaglandin system, and 4) the kallikrein-kinin system. In addition, non-related studies have concerned the agents responsible for bronchial asthma, the role of cyclic AMP in parathyroid function, and the chemical mediators of neural transmission and of taste sensation.

1) Studies on the autonomic nervous system have included studies in enzymes in tissues, physiologic studies in animals, and clinical studies in man. The first and limiting step in biogenesis of catecholamines is hydroxylation of tyrosine by tyrosine hydroxylase. This is a mixed function oxygenase requiring tetrahydrobiopterin as its natural cofactor. We have shown that this enzyme functions at about 1% of its total potential in rat corpus striatum in vivo and that this almost complete inhibition depends upon end-product inhibition by dopamine as well as limitation of quanities of tetrahydrobiopterin in brain tissue. Neuroleptic drugs and lesions in the nigro-striatal area greatly increase the rate of tyrosine hydroxylation in vivo without change in the concentration of cofactor or of dopamine. It was shown that the affinity of tyrosine hydroxylase for cofactor was increased by these interventions. It was shown by chromatography of the active enzyme on G-25 Sephadex that the alteration in affinity results in turn from a change in macromolecular structure. It was further shown that the activation of the enzyme depends in turn upon cAMP-dependendent protein phosphorylation; such activation is totally dependent upon a source of ATP and partially dependent upon cAMP and $Mg^{++}$. It has not been demonstrated thus far that phosphate is incorporated in the enzyme under these conditions; however, since conditions inducive to phosphorylation increase activity of tyrosine hydroxylase 8- to 10-fold in the presence of physiologic concentrations of dopamine and reduced pterin, a phosphorylated intermediate probably exists. Results suggests that such phosphorylation of enzyme is important for the normal regulation of neurotransmitter synthesis. These studies provide a molecular explanation of the action of neuroleptic drugs.

Tyrosine hydroxylase production is regulated by trans-synaptic induction, and appears to depend specifically on the ratio of cAMP to cGMP. A number of pharmacologic agents have been shown to change this ratio. The induction of tyrosine hydroxylase in autonomic ganglia is stimulated by carbonydrate-active steroids and the receptors for these have been studied. Induction by dexamethasone, for example, is blocked by 11-desoxycortisol which has affinity for the same receptors. It was found also that beta receptor agonists can induce the enzyme when steroid medium is kept constant.

Dopamine-beta-hydroxylase (DBH) is the final enzyme in the biosynthetic pathway of norepinephrine. It was previously shown in this laboratory that this molecule consists of 4 subunits, each with a molecular weight of 75,000

daltons, and that the enzyme is a glycoprotein containing about 5% carbohydrate. Since the protein is fully active when bound to the lectin concanavalin A, but not when bound to antibody, it appears that the carbohydrate is remote from the active site and may serve to orient the enzyme in the vesicular membrane. Peptide maps of DBH following cleavage with cyanogen bromide show 11 major peptides. The 4 subunits are probably identical, each containing 12 methionines. These amino acid analyses of DBH differ significantly from those in the literature.

DBH is a copper-containing mixed function oxygenase which normally accepts electrons from ascorbate with a redox potential +200mV. A major advance has been the development of a cell-free wheat germ system which will measure the specific translation of DBH from adrenal polysomes, thus enabling the study of DBH synthesis in vitro.

DBH is released during sympathetic nerve transmission by exocytosis, and circulates as subunits of molecular weight 75,000. As the release of DBH by monocytosis from synaptic vesicles always parallels the release of norepinephrine from the same site, circulating DBH has been studied as an index of norepinephrine release. The cerebrospinal fluid was examined for DBH with the use of a radioimmunoassay; the enzyme is undetectable in CSF.

Plasma DBH is generally high in pheochromocytoma; following successful surgical intervention, plasma concentrations decrease with a half life of 8 hours. Further clinical studies with DBH show a small, insignificant increase in circulating DBH when normal subjects assume the upright posture; this parallels similar changes in circulating norepinephrine and suggest that neither measure provides the index to the activation to the adrenergic nervous system withstanding. The circadian variation in plasma DBH with normal activity showed small, statistically significant changes of 5% of the mean which are physiologically of no importance. Plasma DBH can be depressed in normal man by rapid infusion of albumin (the depression exceeding the dilution effect). In hypertensive subjects, excretion of epinephrine or norepinephrine on a low-sodium diet was significantly greater than that by normals; studies are underway to measure the circadian variations of these pressors in normals and hypertensives.

The role of nerve impulses in blood pressure control was studied by measurement of incorporation of lysine into non-collagen protein of blood vessel walls in spontaneously hypertensive rats. Such incorporation is excessive in these rats as compared to normal ones. Whereas this augmented incorporation could not be inhibited by control of blood pressure with the vascular dilator apresoline, it could be prevented by ganglionic blockade with hexamethonium. These results suggest that nerve impulses and not high pressure alone are necessary for the increased vascular smooth muscle synthesis which occurs with elevated blood pressure.

2) Control of renin release was studied in animal models and in man. In vitro cultured endothelial cells were found to produce an enzyme which can produce angiotensin II from renin substrate, and also from angiotensin I. Studies are underway to purify the enzymes involved, which are different from known converting enzyme, and which may provide an alternative pathway for release of angiotensin II. The mechanisms for the increase secretion of renin

following sinoaortic denervation and vagotomy were further pursued. It is apparent that vagal afferent impulses tonically inhibit autonomic efferent impulses to the kidneys and the blood vessels, and that such efferent impulses control renin release. Extensive studies were begun on the syndrome of low renin hypertension to elucidate the mechanism for renin suppression; these are described below.

3) Prostaglandins: Because prostaglandins of the E series (PGEs) are vasodilators, we have studied these agents to determine whether deficiencies in their production may lead to hypertension (PGFs are pressor agents which might lead to hypertension directly). We have studied them by their administration to animals and by the inhibition of prostaglandin synthetase in animals and in man.

Chemically, the prostaglandins are formed intracellularly from fatty acid precursors; for example, arachidonic acid released by a phospholipase from the plasma membrane is converted by microsomes to the potent $PGG_2$ and $PGH_2$ which are in turn rapidly reduced to the relatively weak prostaglandins $PGF_{2\alpha}$ and $PGE_2$. We have developed radioimmunoassays which will measure PGE in female urine (results in male urine depend largely upon the contribution of the reproductive tract). We have also measured $PGA_2$, produced by dehydration of $PGE_2$.

We have discovered two new prostaglandins formed by addition of glutathione (GSH) to carbon-11 of PGA, and by enzymatic reduction of the 9-keto group in PGA-11-GSH. This conjugate is found in human female urine and constitutes the first clear evidence that PGA may be produced in substantial quantities in the human kidney.

It has been established from previous work in this Branch that PGE infused into the renal artery increases sodium excretion and increases free water clearance, effects attributed to a decrease of sodium reabosrption in the proximal tubule, possibly related to the vasodilator effects of PGE. We have shown also that when indomethacin, an inhibitor of prostaglandin synthetase, is infused into the renal artery, this causes an increase in sodium excretion and a decrease in free water clearance. These effects are attributed to a decrease by indomethacin of sodium reabsorption in medullary areas, where sodium reabsorption increases free formation. This indicates that prostaglandins themselves stimulate sodium reabsorption in medullary areas.

It was found that PGE excretion is less by the kidney in which renal artery stenosis has been produced experimentally than by the normal kidney.

Clinical studies with prostaglandins have shown that assumption of the upright posture results in an increase in urinary PGE of about 20%. In patients with renal artery stenosis, excretion of PGE on the side of the stenosis is lower than that on the normal side.

It was found that infusion of PGE into the carotid artery stimulates secretion of vasopressin; in the water-loaded dog, this results in decrease in free water excretion. Thus PGE is another substance capable of releasing vasopressin; studies are underway to determine its role in physiological control of ADH.

4) Kinins: Plasma pre-kallikrein is a circulating protein which may be converted by activated Hageman factor or other activators to plasma kallikrein, whose molecular weight is about 100,000. There are 3 circulating inhibitors of plasma kallikrein.

Kallikrein can liberate bradykinin, and lysylbradykinin (kallidin) from circulating kininogen. Plasmin, which can be liberated from circulating plasminogen, also has affinity for the kininogens and can release kinins from them. Since bradykinin is a potent vasodilator, this system is under study to determine whether elevation of blood pressure might depend in part upon a deficiency of kinins.

Human urine also contains kallikrein which is different from circulating kallikrein, and whose function is unknown. We have shown earlier that its excretion increases with sodium-retaining steroids and in primary aldosteronism and is decreased in idiopathic hypertension. Recent evidence suggests that kidney kallikrein may enter the blood pari passu with its entry into urine.

Two highly purified kininogens $B_2$ and $B_4B$, major components of the high and low molecular weight kininogens, respectively, have been prepared and subjected to kinetic studies. Human plasma kallikrein has a much higher affinity for $B_4B$, whereas urinary kallikrein has a higher affinity for $B_2$. $B_2$ was shown to correct the coagulation defect known as Flaujeac, Williams, or Fitzgerald trait; $B_4B$ does not.

Human urinary kallikrein was further studied. After treatment with diisopropylfluorophosphate (DFP) it can no longer liberate kinin from kininogen Type II which contains the kinin moiety within the polypeptide chain, but can still release kinin from type I in which the kinin is at the C-terminal end. These results suggests that human urinary kallikrein contains two catalytic sites, and further that HUK is unique among kallikreins in lacking a serine residue at the active site which cleaves the methionyl-lysyl bradykinin as well as lysylbradykinin (Kallidin), and bradykinin. As urinary kallikrein will not produce methyl-lysylbradykinin, the action of another enzyme such as uropepsinogen in the kidney is probably responsible for each cleavage from kininogen.

In clinical studies on human urinary kallikrein it was found that potassium loads which stimulate aldosterone secretion also stimulate the release of kallikrein from the kidneys. It was found that urinary kinin excretion is significantly correlated with urinary kallikrein excretion, suggesting an action of urinary kallikrein on substrate within the kidney. The quantity of methyl-lysylbradykinin in urine may exceed that of lysylbradykinin or of bradykinin; women excrete only 10% as much bradykinin as men. It has also been found that blacks excrete less kallikrein than whites, a finding which correlates with the relative incidence of hypertension in the two groups. Urinary kallikrein is lower in the urine from the stenotic kidney in patients with renal artery stenosis; production of renal artery stenosis in dogs and rats likewise lowers the kallikrein excretion from the treated side. Changes in urinary kallikrein are related in these experiments to changes in renal blood flow, but not to changes in urine volume or in urinary sodium and potassium.

Clinical studies: With the Clinical Pharmacology Section of the Clinical Pharmacology Branch, we have instituted a screening program to define the characteristics of patients with hypertension and to study the pharmacology and pharmacologic kinetics of blood pressure-lowering agents.. Hypertensives are classified according to salt sensitivity and renin secretion. Patients in each group are studied for the presence of sodium-retaining steroids other than aldosterone which might explain the syndrome. This involves the production of tracer steroids and determination of secretion rates. Two patients with low renin hypertension were treated with aminoglutetimide , an inhibitor of adrenal steroid synthesis, with a resultant fall of 20-30 mmHg in systolic and diastolic blood pressure after 10 days. The steroid patterns are being determined again during suppression. Other patients were subjected to suppression of steroid action with aldactone (spironolactone). This has proved successful therapy of hyperaldosteronism and of diagnostic value to determine dependency of the hypertension upon sodium-retaining steroids.

In view of the gynecomastia and loss of libido which commonly accompany treatment with spironolactone, we have studied the effects of this agent. In studies carried on with the Laboratory of Chemical Pharmacology, we have shown that spironolactone destroys microsomal cytochrome P450 in the testis, and thus lowers 17-alpha-hydroxylase activity required for the production of 17-alpha-hydroxyprogesterone, androstenedione, and testosterone.

A comprehensive in-patient clinical study was carried out with the dual objective of defining the accompaniments of hypertension, and of studying the circadian interrelations of blood pressure and the various factor with known or suspected relationship to blood pressure. Of fourteen hypertensive subjects studied on 7-day periods of low, normal and high sodium intake, 9 were found to have striking increases of systolic blood pressure with increase of sodium intake. In the data from the non-salt-sensitive hypertensive subjects and those from the normal subjects, compared with those from the salt-sensitive hypertensive patients, it was found that the salt-sensitive hypertensive patients had higher 17-hydroxycorticosteroid excretion on all three sodium intakes. The factors responsible for this salt sensitivity are under investigation. The circadian variations of 17-hydroxycorticosteroids and of aldosterone excretion showed the same peaks and troughs on all three intakes; the amplitude of the variations of blood pressure were higher the higher the salt intake. Plasma renin activity in both groups of hypertensive patients was higher than normal at all times of day on the low-sodium intake. All of 14 patients showed circadian variations in plasma prekallikrein, with peaks at 4am and 8am on the 9 and 109 mEq sodium intake. Two patients with primary aldosteronism had higher values for plasma prekallikrein at all times of day than did the patients with "essential" hypertension.

Studies of the function of the adrenergic nervous system in hypertension revealed that when hypertensive patients are given low-sodium intake they excrete more sodium and "norepinephrine plus epinephrine", than do normal subjects on the same regimen. The greater than normal excretion of norepinephrine and epinephrine suggest the presence of hyperresponsive adrenergic nervous systems in these hypertensive patients.

Patients with the syndrome of juxtaglomerular hyperplasia, hypokalemia, alkalosis, aldosteronism, and elevated plasma renin activity paradoxically

show normal blood pressure even with expansion of vascular volume. We have found that "basal" prostaglandin excretion in patients with this syndrome were higher than normal. Two such patients were treated with indomethacin, an inhibitor of prostaglandin synthetase, with resultant increase in plasma potassium, retention of sodium, and decrease in urinary PGE. Plasma renin activity, elevated in the control periods, fell to normal during treatment with indomethacin. Metabolic studies are being carried out to define the effect of indomethacin in this syndrome on aldosterone, prostaglandins, prekallikrein, and kallikrein. It is possible that excessive production of vasodilators (PG's, kinins) balances the excessive production of angiotensin and aldosterone in this syndrome to produce the persistently normal blood pressure.

Studies of calcium and phosphorus metabolism in relation to metabolic bone disease, parathyroid function, and the formation of renal stones: Studies which relate nephrogenous 3,5'cyclic AMP to various abnormalities of parathyroid function have been extended to normal parathyroid physiology, and to patients of hypercalciuria with unknown etiology. From the data available from 10 patients with hypoparathyroidism, 35 normal subjects, and 39 patients with hyperparathyroidism, it was concluded that measures of nephrogenous cyclic AMP give a sensitive and reliable method for study of the spectrum of parathyroid disease.

Patients with nephrolithiasis and increased gastrointestinal calcium absorption have been found to respond to treatment with sodium cellulose phosphate, an agent with inhibits calcium absorption from the gut. In this study, designed to evaluate long-term effects of this drug, it has been shown that no new stones are formed and no metabolic bone disease has been produced by the treatment (1-5 years in duration), nor has there been evidence of trace metal depletion. The expected secondary hyperparathyroidism has not been thus far apparent.

Other studies: The chemisty and control of tryptophan hydroxylase has been studied in extenso. This enzyme requires tetrahydrobiopterin and is a mixed-function oxygenase. Its distribution in the central nervous system is limited to serotonergic neurons, and these were selectively destroyed by administration of 5,6- and 5,7-dihydroxytryptamine (DHT) which are specifically taken up by these neurons and subsequently destroy them. Intracerebral administration of 5,7-DHT to neonatal animals results in immediate loss of tryptophan hydroxylase in all brain regions, and prevents normal developmental increase in the enzyme. The growth rates of rats so treated is markedly retarded, despite normal concentrations of circulating growth hormone. Studies show that the neurotoxicity of these dihydroxyindoles depends upon their tight binding to mitochondria,and presumably their interuption of the respiration of the cell.

It was shown that parachloroamphetamine and methyl parachloroamphetamine, which deplete brain serotonin, also deplete brain tryptophan hydroxylase, and that this depletion lasts for 3 weeks after a single dose. It appears that nerve ending regions of the brain are initially destroyed, with retrograde death of the cell body. The initial event appears to be the specific uptake of the drug by serotonin nerve ending.

Studies have continued on the identification of mediators released from human lung by antigen-antibody reactions. A greatly simplified method has been

developed for the measurement of histamine. In addition to histamine, an arginine esterase, SRS-A, and prostaglandins are also released when passively sensitized human lung is reacted with specific antigen. Human lung releases mainly prostaglandin E's which relax human bronchi, and it appears unlikely that prostaglandins have a direct mediator effect in bronchial asthma. However, they may act as modulators of this disease either by potentiating the effects of other mediators or by altering levels of cyclic AMP. The arginine esterase released from human lung is not a kallikrein, an activator of plasma kallikrein or plasmin or a plasminogen activator. The enzyme is unusually labile to salts, losing 50% of its activity on standing in 0.2 M NaCl for one half hour at room temperature. A method has been devised for the purification of this enzyme with a 46% yield. Methods have been devised for the separation of human SRS-A's into four biologically active fractions. The four fractions inhibited type H-1 arylsulfatase at pH 4.5. At this pH and at ratios of arylsulfatase units/SRS-A units of 0.3 to 6.0, the arylsulfatase did not destroy the biological activity of the SRS-A's except for Fraction 1. These and other results suggest that human SRS-A's, like the prostaglandins, may represent a family of compounds. Studies have been initiated on the biochemical mechanism of the synthesis of SRS-A in monkey lung. The conditions required for optimal release of mediators from monkey lung are similar to those required for human lung, although higher concentrations of antigen E and/or longer periods of incubation are required. The amount of histamine and SRS-A released from monkey lung is similar to that of human lung but only one-tenth of the arginine esterase activity is released. Studies of SRS-A formation in monkey lung homogenates have been difficult to interpret, since homogenates contain substance(s) other than histamine and SRS-A which contract the guinea pig ileum. Preliminary experiments indicate the feasibility of separating SRS-A from the interfering materials.

Separation of all but two of the 20 amino acid phenylthiohydantoins has been achieved by high performance liquid chromatography. This procedure is currently in use in the amino acid sequence analysis of peptides and proteins. The phenylthiohydantoins also have been separated by a less expensive and potentially more rapid isocratic method employing two separate columns. Dinitrophenyl derivatives of amino acids have been separated by a similar procedure which is more rapid, sensitive and specific than previous methods. The technique is currently employed in the N-terminal amino acid analysis of polypeptides. Tryptophan, peptides containing N-terminal tryptophan, tryptamine and certain related indoles react with Fluram to form derivatives with uniquely high fluorescence in strong acid. Fluram also has been used to develop a membrane filter assay for proteins in the submicrogram range.

A zinc protein from parotid saliva has been isolated from human subjects with normal taste acuity by gel filtration and ion exchange chromatography. The protein has a molecular weight of 37,000 and does not appear to have subunits. It is composed of 8% histidine residues and has 2 moles of zinc per mole of protein. The contractile mechanism has been described in the taste buds of fungiform papillae, and acetylcholinesterase has been found in the pore region of the taste buds from circumvallate papillae of rats. Radioactive sugars have been demonstrated to bind in a specific manner to membranes isolated from taste buds of circumvallate papillae; they do not bind to non-taste bud bearing membranes from the epithelial tissue surrounding these papillae.

A double blind study of the effects of zinc sulfate and of placebo in a group of 106 unselected patients with taste and smell dysfunction was carried out. The results indicated placebo and zinc sulfate were effectively equivalent in the treatment of these disorders. The clinical and pathophysiologic characteristics of patients with post-influenzal hypoguesia and hyposmia have been evaluated. Biopsy of the nasal mucous membrane shows inflammation of the upper lamina propria in such patients.

A new syndrome of acute zinc depletion has been elucidated. In addition to changes in several sensory modalities these patients suffer from severe cerebellar dysfunction including intention tremor, positive Romberg sign and ataxia. Treatment with zinc ion corrects these abnormalities within 24-48 hours.

Human pituitary hormones have been grown in vitro in capillary tissue culture. These tumors have been maintained for several months with production of large amounts of growth hormone and of prolactin. These hormones have been characterized by physical, chemical and biological techniques which show them to be indistinguishable from normal human hormones.

The system whereby the sympathetic nervous system (cervical sympathetic nerves) can induce serotonin N-acetyltransferase in the pineal gland was further studied. Nerve impulses appear to mediate production of cyclic AMP in the pineal; large quantities of cyclic AMP-dependent protein kinase in the pineal suggest that the next step is phosphorylation of chromatin-related protein followed by transcription of the messenger RNA for serotinin N-acetyltransferase. A number of protein kinases have been isolated in association with ribosomes; their role in this system is under study.

Project No. Z01 HL 01801-01
1. Hypertension-Endocrine Branch
2. Steroid & Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Outpatient Hypertension Diagnostic Screening Program

Previous Serial Number:

Principal Investigators: Taylor, A. A., M.D., Ph.D.
Mitchell, J. R., M.D., Ph.D.
Keiser, H. R., M.D.
Bartter, F. C., M.D.

Other Investigators: Snodgrass, W. R., M.D., Ph.D.
Horwitz, D., M.D.
Licata, A. A., M.D., Ph.D.
Vinci, J., M.D.
Gill, Jr., J. R., M.D.
Delea, C. S.

Project Description:

An estimated 23-24 million Americans or approximately 10% of our population have hypertension.

Significant improvements in the ability to diagnose and to cure certain forms of hypertension have occurred through the utilization of increasingly more sophisticated biochemical tests which permit more accurate categorization of hypertensive patients into selected subgroups. The biochemical tests important in the diagnostic classification of hypertensive patients have been available in various laboratories in the Hypertension-Endocrine Branch but an organized application of these tests to the screening of large numbers of outpatient hypertensives has not been instituted previously. This project was designed to categorize hypertensive patients into established subgroups in order to study various characteristics of such subgroups more intensively including their response to different types of drug therapy.

Project Protocol: Each patient referred to the Hypertension outpatient clinic is seen by a physician who takes a history and performs a physical examination. A chest x-ray, electrocardiogram, urinalysis, urine culture and routine serum, chemistries are obtained during the patient's first visit. An intravenous pyelogram and radioactive renogram are obtained between the first and second visits. Each patient is taught to take his own blood pressure and is requested to take it 6 times a day. On the morning of the second visit, the patient brings a 24 hr urine sample for sodium,

potassium, creatinine, 17OHCS, 17KS and aldosterone excretion rate, and 45 min.-supine and 3 hour- upright blood samples are obtained for plasma renin activity, aldosterone and cortisol. The same protocol is followed on the third visit as the second except that each patient is given Lasix 40 mg after the first blood sample is obtained.

Based on these data the patients is then placed in a diagnostic category and he is included in one of several ongoing protocol studies if appropriate.

Consenting normotensive volunteers are studied in a similar fashion except that they are not given Lasix.

Major Findings: Since December 1974, 25 normotensive volunteers (age range 19-61 y.o.) and 70 hypertensive patients (age range 15-74 y.o.) have been or are being studied. Since initiating this study there have been no fatalities or morbid cardiovascular or neurovascular events. Among this patient population are one person with primary aldosteronism, one patient with suspected renovascular hypertension, one patient with coarctation of the aorta, one patient with supravalvular aortic stenosis, and 8 of 34 (24%) patients with suppressed plasma renin activity (upright plasma renin activity 2 ng/ml/hr. after 40 mg Lasix p.o.). No patients with pheochromocytoma have been identified. Thirty of 45 patients who have completed the screening studies are or have agreed to participate in further diagnostic or therapeutic studies. Only one patient with spontaneous hypokalemia has been identified. Many of our patients have come from the NIH employee population.

Keyword Descriptors:

Hypertension screening, renin-angiotensin-aldosterone, normal volunteers

Honors and Awards:

Publications:

Project No. Z01 HL 01802-01
1. Hypertension-Endocrine Branch
2. Steroid & Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Adrenal steroid secretion in hypertension.

Previous Serial Number:

Principal Investigator: Mitchell, J. R., M.D., Ph.D.

Other Investigators: Taylor, A. A., M.D., Ph.D.
McMurtry, R. J., M.D., Ph.D.
Bartter, F. C., M.D.

Cooperating Units:

Project Description:

To investigate the control mechanisms for secretion of adrenocortical steroids and their roles in the genesis of differnet types of hypertension.

Several studies have shown that about 20% of patients with hypertension have suppressed plasma renin activity (Woods et al., 1969; Jose, et al., 1970). Only a small number ( 1-2%) of these have primary hyperaldosteronism. It has been suggested by several investigators that the hypertensive state in the remaining patients can be attributed to excessive secretion of some other unknown adrenal steroid having minerlocorticoid activity because: 1) they have low renin activity; 2) administration of inhibitors of adrenal steroid biogenesis, such as aminoglutethimide, ameliorates their hypertension but not that of hypertensives with normal renin; 3) administration of spironolactone, an antagonist of mineralocorticoid actions, lowers their blood pressure; and 4) bilateral adrenalectomy lowers their blood pressure. Three groups have looked at individual steroids in these patients. Brown et al., 1972 have reported elevated plasma desoxycorticosterone concentrations in 6 of 31 hypertensive subjects, and all 6 had suppressed plasma renin activity. Melby et al., (1971) noted 3 of 12 patients with "low renin" hypertension had elevated 18-hydroxy-desoxycorticosterone secretion rates. Sennett et al., (1974) have recently reported that 15 of 15 patients with "low renin" hypertension and 1 of 15 patients with essential hypertension have elevated 16-B-hydroxy-dehydroepiandrosterone excretion rates. However, in none of these studies has a profile of several adrenal steroid secretion rates been examined.

Methods Employed

Patient with hypertension categorized as essential, low renin, hyperaldosterone or renovascular in type will be studies under 3 different regimens; 1) Low sodium intake (9 mEq sodium, 70 mEq potassium for 1 week), 2) Normal sodium intake (109 mEq sodium, 70 mEq potassium for 5 days), 3) Normal sodium intake, ACTH stimulation [250 ug tetracosactrin (synthetic B 1-24 ACTH) intravenously over 8 hr. and urine and plasma collected for 2 days]. During each regimen urine secretory rates will be determined for the following steroids by administration of radioactive tracer doses intravenously followed by double isotope dilution assay of urine metabolites: $^{3}$H-dehydroepiandrosterone ( 1 ug, 4 uCi), $^{3}$H-dehydroepiandrosterone sulfate ( 1 ug, 4 uCi), $^{3}$H-16-B-hydroxydehydroepiandrosterone sulfate ( 1 ug, 4 uCi), $^{3}$H-16-u-hydroxy-dehydroepiandrosterone sulfate ( 1 ug, 4 uCi), $^{3}$H-18-hydroxy-corticosterone ( 1 ug, 4 uCi), $^{3}$H-18-hydroxy-desoxycorticosterone ( 1 ug, 4 uCi), $^{3}$H-17 -hydroxy-pregnenolone ( 1 ug, 4 uCi). In addition, 5 plasma samples (10 ml each) will be obtained and urine will be collected for 48 hr. Plasma DOC and 17- hydroxy-progesterone will be determined by radioimmunoassay and urine excretion of metabolites of cortisol, desoxycortisol, corticosterone and aldosterone will be assayed chemically or by radioimmunoassay.

The initial two patients in each of the hypertension groups will be given four of the $^{3}$H-steroids and the secretory rates determined; 2 days later the remaining three $^{3}$H-steroids will be administered and their secretory rates determined. If as anticipated, the metabolites of the various steroids do not interfere with the assay of the other steroids, all subsequent patients will receive the seven $^{3}$H-steroids simultaneously for concomitant evaluation of secretory rates.

Major Findings

Currently 6 patients, 4 with low renin essential hypertension and 2 with normal renin essential hypertension have or are participating in this study. Adrenal steroid secretory data are incomplete at this time.

Keyword Descriptors:

hypertension, primary aldosteronism, renovascular hypertension, "low renin" hypertension, adrenal steroid secretion

Honors and Awards:

Publications:

Project No. Z01 HL 01803-01
1. Hypertension-Endocrine Branch
2. Steroid & Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Aminoglutethimide in low renin essential hypertension.

Previous Serial Number: None

Principal Investigator: Mitchell, J.R., M.D., Ph.D.

Other Investigators: Taylor, A., M.D., Ph.D.
Gill, Jr., J.R., M.D.
Snodgrass, W.R., M.D., Ph.D.
McMurtry, R.J., M.D., Ph.D.
Dybing, E., M.D.

Cooperating Units:

Project Description:

Patients will be tested for the blood pressure-lowering effect of aminoglutethimide, an inhibitor of adrenal steroid synthesis. The pathways of adrenal steroid synthesis that are inhibited by aminoglutethimide in blood-pressure responsive patients, and therefore the pathways that might be mediating the hypertension, will be determined.

Treatment with aminoglutethimide lowers the blood pressure of patients with hypertension secondary to primary aldosteronism or Cushing's syndrome (Gaunt, et al., Clin. Pharmacol. Therap. 9: 657, 1968; Temple and Liddle, Ann. Rev. Pharmacol. 10: 199, 1970; Fishman, et al., J. Clin. Endo. Metab. 27: 481, 1967; and Gorden, et al., J. Clin. Endo. Metab. 28: 921, 1968). In 1969 Liddle's group (Woods, et al., Arch. Int. Med. 123: 366, 1969) reported that 6 of 9 hypertensive patients with the syndrome of low-renin (normal aldosterone) essential hypertension experienced a lowering of blood pressure after administration of aminoglutethimide but patients with normal renin hypertension failed to respond. They noted also that the hypertension of low-renin patients responded to therapy with spironolactone (Carey, et al., Arch. Int. Med. 130: 849, 1972), a renal antagonist of sodium-retaining steroids, and to bilateral adrenalectomy (Gunnels, et al., Ann. Int. Med. 73: 901, 1970). This report triggered a widespread search for the excessive adrenal secretion of a sodium-retaining steroid other than aldosterone, and several groups subsequently have

postulated that various steroids are of etiologic significance in low-renin hypertension (Brown, et al., Lancet ii: 243, 1972; Melby, et al., Circ. Res. 23: II-143, 1971; Kuchel et al., Circ. Res. 23: II-150, 1971; Messerli, et al., Proc. 56th Meeting of Endocrine Society, p. A-63, 1974; Slaton, et al., Clin. Res. 23: 45A, 1975; and Hisahsatu, et al., J. Clin. Endo. Metab. 40: 156, 1975). However, in these studies only the particular steroid of interest was examined. No attempt was made to determine the secretion rates of most other adrenal steroids nor to demonstrate cause-effect relationships between increased steroid secretion and elevation of blood pressure. Indeed, the direct involvement of the proposed steroids in the pathogenesis of low-renin hypertension can be questioned, since none of the steroids have sufficient intrinsic sodium-retaining activity to be physiologically effective in the amounts apparently secreted by patients with low-renin hypertension.

The antihypertensive effects of aminoglutethimide result from its inhibition of adrenal steroid secretion and the accompanying renal loss of sodium and water; the drug has no direct effect on the kidney, the adrenergic nervous systme or vascular smooth muscle in adrenalectomized animals and people (Gaunt, et al., Clin. Pharmacol. Therap. 9: 657, 1968; Temple and Liddle, Ann. Rev. Pharmacol. 10: 199, 1970; Fishman, et al., J. Clin. Endocr. Metab. 27: 481, 1967; Gorden, et al., J. Clin. Endocr. Metab. 28: 921, 1968; Woods, Liddle, et al., Arch. Int. Med. 123: 366, 1969). Thus, the secretion of a sodium-retaining adrenal steroid, be it aldosterone or an unidentified steroid, should play an important pathogenetic role in the hypertension of low-renin patients whose blood pressure responds to aminoglutethimide therapy.

Careful review of numerous biochemical studies in dogs and other animals, with sufficient clinical studies to confirm that man responds similarly, reveals that the secretion of cortisol by the adrenal zona fasciculata is only minimally affected by aminoglutethimide in endocrinologically normal subjects because of a compensatory increase in ACTH secretion (Gaunt, et al., Clin. Pharmacol. Therap. 9: 657, 1968; Temple and Liddle, Ann. Rev. Pharmacol. 10: 199, 1970; Fishman, et al., J. Clin. Endocr. Metab. 27: 481, 1967; Gorden, et al., J. Clin. Endocr. Metab. 28: 921, 1968; and Woods, Liddle et al., Arch. Int. Med. 123: 366, 1969). In contrast to the cortisol pathway, secretion of aldosterone by the adrenal zona glomerulosa remains inhibited by aminoglutethimide even after months of continuous therapy and compensatory increases in renin do not overcome the inhibition (Gaunt, et al., Clin. Pharmacol. Therap 9: 657, 1968; Temple and Liddle, Ann. Rev. Pharmacol. 10: 199, 1970; Fishman, et al., J. Clin. Endocr. Metab. 27: 481, 1967; Gorden, et al., J. Clin. Endocr. Metab. 28: 921, 1968; and Woods, Liddle et al., Arch. Int. Med. 123: 366, 1969).

This phenomenon of preferential inhibition of aldosterone synthesis provides a powerful tool for defining the physiologic role of the steroids postulated as having etiologic significance in the genesis of low-renin hypertension. If the secretion of a particular steroid is not inhibited

by aminoglutethimide therapy when blood pressure is reduced, then that steroid cannot be solely responsible for the hypertension. By the same rationale, one can determine which adrenal steroid synthetic pathways are inhibited by aminoglutethimide in blood pressure-responsive patients and therefore which pathways might be mediating the hypertension.

Patients with low-renin hypertension on a normal diet will be hospitalized for 3 days and secretory rates will be determined for the following steroids by administration of radioactive tracer doses intravenously followed by double-isotope dilution assay of urinary metabolites: $^{3}$H-dehydroepiandrosterone ( 1 ug, 4 uCi), $^{3}$H-dehydro-epiandrosterone sulfate ( 1 ug, 4 uCi), $^{3}$H-16 B-hydroxy-dehydro-epiandrosterone ( 1 ug, 4 uCi), $^{3}$H-16-B-hydroxy-dehydroepiandrosterone sulfate ( 1 ug, 4 uCi), and $^{3}$H-18-hydroxy-desoxycorticosterone ( 1 ug, 4 uCi). Five plasma samples (10 ml each) will be obtained, and urine will be collected for 48 hr. Plasma DOC, corticosterone, desoxycortisol, cortisol, aldosterone, progesterone and 17- -hydroxy-progesterone will be determined by radioimmunoassay and urinary excretion of metabolites of cortisol and aldosterone will be assayed chemically or by radioimmunoassay.

Following these control determinationas, patients will begin treatment with aminoglutethimide ( 1 g per day in divided doses) and will be observed closely for clinical evidence of acute cortisol insufficiency. Plasma cortisol and aldosterone and urinary free cortisol will be monitored. Aminoglutethimide has an immediate onset of action, and cortisol insufficiency would quickly be apparent. Accordingly, asymptomatic patients will be discharged after 4 days of treatment and be followed weekly in the clinic for another 14 days. As outpatients, they will record their blood pressure several times daily under the normal conditions of their environment. At each weekly clinic visit, blood (20 ml) will be obtained for electrolytes, CBC, SGOT, glucose, urea nitrogen, aldosterone, cortisol and renin. Patients will then be hospitalized for a final 3 days and the steroid secretory and excretory determinations will be repeated exactly as above (total duration of treatment with aminoglutethimide = 3 weeks).

Major Findings: Aminoglutethimide has been administered to two patients with low renin essential hypertension. In both subjects, control blood pressures of 150/100 and 140/95 had fallen to mean values of 110/70 and 115/75 after 10 days of therapy. There was an associated fall in aldosterone excretion rate from control values of 3.99 ug/24 hr. to 1.49 ug/24 hr. in one patient in whom the data has been analyzed. Adrenal steroid secretory rate data are incomplete at this time.

Keyword Descriptors:

aminoglutethimide, low renin essential hypertension;
adrenal steroid secretion

Honors and Awards:

Publications:

None.

Project No. Z01 HL 01804-01
1. Hypertension-Endocrine Branch
2. Steroid & Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Effects of Spironolactone

Previous Serial Number:

Principal Investigator: Taylor, A. A., M.D., Ph.D.

Other Investigators: Mitchell, J.R., M.D., Ph.D.
McMurtry, R.J., M.D., Ph.D.
Snodgrass, W.R., M.D., Ph.D.
Dybing, E., M.D, Ph.D.
Bartter, F.C., M.D.

Cooperative Units: None

Project Description:

To examine the effects of spironolactone or renin-angiotensin-aldosterone physiology and on sex steroids in hypertensive patients.

Spironolactone is widely used as a potassium-conserving diuretic and antihypertensive agent. It has been suggested by some as the drug of choice in patients with low renin essential hypertension, a subgroup comprising approximately 25% of the hypertensive population. Previous work by some of us has shown that spironolactone destroys cytochrome P-450 in the adrenal and testis of certain experimental animals (Menard et al., Endo. 94: 1628, 1974; Menard et al., J. Steroid Biochem. 5: 365, 1974). Cytochrome P-450 is a co-enzyme necessary for normal function of the steroid 17-hydroxylase enzyme; 17-hydroxylase activity is decreased in experimental animals given spironolactone. A recent study of the effect of 400 mg spironolactone per day for five days in normal male volunteers showed only a transient rise in plasma LH and FSH but no change in plasma testosterone, estradiol, or prolactin. These findings failed to explain the frequently observed side effects of gynecomastia, decreased libido, and impotence in male hypertensives taking spironolactone.

Significant interference by spironolactone with parameters of the

renin-angiotensin-aldosterone system in low renin hypertensive patients is demonstrated by the recent report of Lowder et al., Liddle (NEJM 29: 243, 1974), that the increased upright plasma renin activity induced by spironolactone in low renin hypertensive patients persisted for 13-36 weeks after discontinuation of the drug.

Patients previously classified as having essential hypertension with normal or suppressed plasma renin activity by diagnostic studies performed while off all antihypertensive medications will be considered for inclusion in the study. Patients with prior history of malignant hypertension, evidence of cardiovascular compromise or severe impairment of renal function (creatinine clearance = 70 cc/min or less) will be excluded. Informed consent will be obtained from all patients. All patients will be provided with equipment and taught to take their blood pressure. Blood pressure will be recorded both by the patient at home and by physicians in the outpatient clinic throughout the study.

Following pretreatment measurements of the plasma and urinary parameters listed below, each patient will receive spironolactone, 400 mg/day, for a maximum of 12 weeks. After 12 weeks of therapy, spironolactone will be discontinued and the patient observed for an additional 6 weeks before reinstitution of antihypertensive therapy unless development of signs and symptoms of cardiovascular compromise or accelerated hypertension necessitate removal from the study and treatment at an earlier time. If there is no reduction in blood pressure after the initial 4 weeks of spironolactone therapy, the patient will be removed from the study and treated with other antihypertensive agents. Before, and at regular 2-4 week intervals during and after discontinuation of spironolactone, the following plasma and urinary measurements will be made: Plasma renin activity, aldosterone, cortisol, deoxycorticosterone, 11-deoxycortisol, testosterone, progesterone, 17-hydroxyprogesterone, 17B-estradiol, prolactin, LH, FSH, Na, K, Cl, $CO_2$, BUN, creatinine, sex hormone binding globulin; urinary creatinine, Na, K, aldosterone, 17-hydroxycorticosteroids, 17-ketosteroids, aldosterone excretion rate.

Currently seven patients have been included in this study, including two patients with primary aldosteronism, one patient with low renin essential hypertension and 4 patients with essential hypertension and normal plasma renin. Only one patient with primary aldosteronism has completed the study. In this patient the pretreatment mean BP was 138 $\pm$ 1.1 mmHg, 95 $\pm$ 1.4 mmHg just prior to discontinuation of spiranolactone therapy and 110 $\pm$ 1.5 mmHg one week later. Upright plasma renin activity was 0.20 ng/ml/hr prior to treatment; 2-21 ng/ml/hr just prior to stopping spironolactone, and 0.08 ng/ml/hr one week after discontinuation of drug therapy. Sex hormone data is currently incomplete.

Keyword Descriptors:

spironolactone, renin-angiotensin-aldosterone

Honors and Awards:

Publications:

Project No. Z01 HL 01805-01
1. Hypertension-Endocrine Branch
2. Steroid & Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Adrenergic nervous system function in hypertension.

Previous Serial Number:

Principal Investigator: Gill, Jr., J.R., M.D.

Other Investigators: Alexander, R.W., M.D., Ph.D.
Keiser, H.R., M.D.

Cooperating Units:

Project Description:

Previous studies in normal subjects indicate that an increase in sodium intake, either rapidly by infusion of saline or slowly by an increase in dietary sodium, results in a decrease in urinary excretion of norepinephrine plus epinephrine and an increase in urinary dopamine. These changes are consistent with a decrease in adrenergic activity and suggests that normally adrenergic activity may be inversely related to the renal formation and excretion of dopamine a potent renal vasodilator and natriuretic catecholamine.

The present studies were designed to extend these observations to include patients with hypertension.

Patients with normal renin essential hypertension were studied on a 9 mEq/day sodium intake for eight days , then on a 249 mEq/day sodium intake for 8 days. Daily collections of urine were analyzed for norepinephrine plus epinephrine (NE+E), dopamine (DA) and sodium.

On a 249 mEq sodium intake values for urinary NE+E and DA in the hypertensive patients were similar to those in the normotensive subjects. When sodium intake was decreased to 9 mEq/day the hypertensive patients excreted significantly more sodium and more NE+E. Dopamine, however, decreased as, in the normotensive subjects, but the values were not significantly different. The results are summarized in the table below.

| Sodium Intake | Patients | $U_{Na}V$ mEq/d | NE+E µg/day | DA µg/day |
|---|---|---|---|---|
| 249 mEq | Normal | 228±15 | 21.1±3 | 195±20 |
| | Hypertensive | 218±8 | 25.1±4.3 | 204±18 |
| 9 mEq | Normal | 37±2 | 37.4±5.3 | 136±18 |
| | Hypertensive | 55±7* | 60.4±7.8* | 161±22 |

*$P < .01$

The results indicate that when hypertensive patients are stressed by a low sodium intake they excrete more sodium and NE+E than normotensive patients. The greater than normal excretion of NE+E, possibly a consequence of the greater sodium loss, suggests the presence of hyperresponsive adrenergic nervous system in hypertension.

Keyword Descriptors:

norepinephrine, dopamine, sodium excretion, adrenergic nervous system, hypertension

Honors and Awards:

Publications:

1. Alexander, R.W., Gill, Jr., J.R., Yamabe, H., Lovenberg, W. and Keiser, H.R.: Effects of dietary sodium and of acute saline infusion on the interrelationship between dopamine excretion and adrenergic activity in man. J. Clin. Invest. 54: 194, 1974.

Project No. Z01 HL 01806-01
1. Hypertension-Endocrine Branch
2. Steroid & Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Pelation of K to vascular response of BP

Previous Serial Number:

Principal Investigator: Radfar, N., M.D.

Other Investigators: Bartter, F. C., M.D.

Cooperating Units:

Project Description:

It is known that the patients with "Bartter's Syndrome" have a vascular hyporesponsiveness to angiotensin II. To investigate the pathogenesis of this resistance, i.e., to see whether it is primary vascular defect or whether it may result from the hypokalemia, it was reasonable to study the vascular response of patients with hypokalemia of diverse origin including patients with JG hyperplasia. Such patients are admitted to the metabolic ward and are put on 109 mEq Na diet and their response to angiotensin is determined during hypokalemia, and after the serum K has returned to normal spontaneously or by K supplementation.

One patient who had diuretic induced hypokalemia has been studied. During hypokalemia the pressor dose required to increase diastolic blood pressure by 20 mmHg was 200 ng/kg/min. When serum K was restored to normal the pressor dose dropped down to 30 ng/kg/min.

Keyword Descriptors:

Bartter's Syndrome, hypokalemia

Honors and Awards:

Publications:

Project No. Z01 HL 01807-01
1. Hypertension-Endocrine Branch
2. Steroid & Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies in Bartter's Syndrome

Previous Serial Number:

Principal Investigators: Bartter, F. C., M.D.
Gill, Jr., J. R., M.D.
Taylor, A. A., M.D., Ph.D.

Other Investigators: Bowden, R., M.D.
Vinci, J., M.D.

Project Description:

Since the initial description of the syndrome of hypokalemic alkalosis, hyperreninism, aldosteronism and juxtaglomerular hyperplasia with normal blood pressure, this branch has maintained an ongoing interest in this disorder. Patients suspected of having the disorder are presently being studied in extenso, including renal biopsy, thorough evaluation of renal handling of Na, K, and water, testing of arteriolar sensitivity to pressor agents, red cell concentration of Na, and evaluation of therapy. Inhibitors of prostaglandin synthetase are being given to evaluate the role of prostaglandins (pg's) in this syndrome. Kinins in serum and urine are also under study.

Nine patients, all female, ages 5 to 40 years, have been evaluated for this syndrome. Two were found to be diuretic abusers and vomiters, three clearly have the syndrome, and three more are still being evaluated for this syndrome. Two were found to be diuretic abusers and vomiters, three clearly have the syndrome, and three more are still being evaluated. The last patient may represent a variant of the disorder in that she has hypokalemic alkalosis, aldosteronism and hyperreninemia, with histologically normal J-G apparatus and normal arteriolar sensitivity to angiotensin II.

Studies in two patients with indomethacin, a prostaglandin synthetase inhibitor, demonstrated high pg's in the control period, low during the therapy with a decrease in plasma renin activity. Both patients had an increase in plasma potassium concentration and retained sodium. More patients are currently being studied under highly controlled conditions.

Keyword Descriptors:

aldosterone, juxtaglomerular hyperplasia, prostaglandin

Honors and Awards:

Publications:

Project No. Z01 HL 01808-01
1. Hypertension-Endocrine Branch
2. Steroid & Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Control of renin: the role of the vagus nerves.

Previous Serial Number:

Principal Investigator: Yun, John C.H., Ph.D.

Other Investigators:

Cooperating Units:

Project Description:

The mechanism for the increase in plasma renin activity (PRA) due to sinoaortic denervation and cervical vagotomy was examined in intact, renal denervated, and renal denervated, adrenalectomized dogs maintained on a high-salt diet.

In eleven intact animals, PRA increased from 2.09 ± 0.76 ng/ml/hr in control periods to 13.09 ± 2.34 ng/ml/hr ($P < 0.005$) 60 minutes after sinoaortic denervation. PRA increased further to 22.82 ± 3.90 ng/ml/hr ($P < 0.005$) 90 minutes after cervical vagotomy. In eight animals previously subjected to renal denervation, PRA increased from 7.33 ± 1.42 ng/ml/hr in control periods to 14.45 ± 2.89 ng/ml/hr ($P < 0.01$) 60 minutes after sinoaortic denervation. Cervical vagotomy in these animals caused a further slight, but not statistically significant, increase in PRA to 16.81 ± 3.77 ng/ml/hr ($P > 0.1$). In six dogs with both renal denervation and adrenalectomy, PRA was 2.07 ± 1.03 ng/ml/hr in control periods, 1.04 ± 0.39 ng/ml/hr 60 minutes after sinoaortic denervation, and 1.89 ± 0.74 ng/ml/hr 90 minutes after cervical vagotomy.

These data suggest that the increase in PRA after sinoaortic denervation is probably due to both increased sympathetic discharge to the kidney and catecholamines released from the adrenal medulla, whereas the increase in PRA after cervical vagotomy is mediated largely by increased sympathetic discharge to the kidney.

Further research will include studies on the mechanism(s) by which renal nerve stimulation causes renin secretion.

Keyword Descriptors:

vagi, renin, renal nerve, sinoaortic nerves, adrenal medulla

Honors and Awards:

Publications:

Project No. Z01 HL 01809-01
1. Hypertension-Endocrine Branch
2. Steroid & Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Renal prostaglandins, sodium and blood pressure.

Previous Serial Number:

Principal Investigators: Gill, Jr., J. R., M.D.

Other Investigators: Alexander, R. W., M.D., Ph.D.
Halushka, P. V., M.D., Ph.D.
Pisano, J. J., Ph.D.
Keiser, H. R., M.D.

Cooperating Units:

Project Description:

Previous studies indicate that the infusion of prostaglandins of the E series into a renal artery produces an increase in sodium excretion. If these effects are similar to those produced by the intrarenal prostaglandins, then inhibition of intrarenal synthesis of prostaglandins should be in association with a decrease in renal sodium excretion. To test this hypothesis, indomethacin, a prostaglandin synthetase inhibitor was infused into a renal artery of the dog.

Methods: Water diuresis was produced in anesthetized hypophysectomized cortisol- treated dogs by infusion of 2.5 per cent dextrose. When urine flow was steady clearance measurements were started. After three control periods were obtained indomethacin 0.35 mg/min was infused into the left renal artery for 100 min followed by post control periods for 2 hours. In a second series of studies, the control period was followed by infusion of Ringer's solution for two hours, then infsuion of indomethacin, was superimposed for one hour. The clearances of inulin ($C_{IN}$) and paraaminohippurate ($C_{PAH}$) were determined. Changes in the clearance of solute-free water ($C_{H_2O}$) were taken as an index of sdium reabsorption by the diluting segment of the nephrons. Changes in the sum of the clearance of solute-free water and the clearance of sodium ($C_{Na}$) per 100 ml glomerular filtration rate (GFR) were assumed to represent changes in

proximal tubular sodium reabsorption. Urinary PGE and PGF like material was determined by radioimmunoassay.

Major Findings: Indomethacin increased sodium excretion ($U_{Na}V$) and decreased $C_{H_2O}$ without apparent effect on $C_{Na} + C_{H_2O}$, $C_{IN}$ and $C_{PAH}$. Infusion of Ringer's increased $C_{Na} + C_{H_2O}$, $C_{H_2O}$ and $U_{Na}V$; the suprimposition of indomethacin produced a further increase in $U_{Na}V$ and decrease in $C_{H_2O}$ but no further change in $C_{Na} + C_{H_2O}$. The results are summarized in the table below.

| | $C_{IN}$ | $C_{Na} + CH_2O$ | $C_{H_2O}$ | $U_{Na}V$ |
|---|---|---|---|---|
| | ml/min | ml/min/100 ml GFR | | μEq/min |
| Control | 33 ± 2.8 | 8.2 ± 1.0 | 7.8 ± 0.9 | 16 ± 2.3 |
| Indomethacin | 35 ± 3.4 | 7.2 ± 0.8 | 5.8 ± 0.6* | 63 ± 7.6* |
| Post control | 35 ± 3.8 | 6.6 ± 1.0 | 5.8 ± 0.9 | 32 ± 8.3 |
| Control | 32 ± 3 | 6.3 ± 0.4 | 6.0 ± 0.4 | 11 ± 2 |
| Ringers | 32 ± 2 | 11.9 ± 0.6 | 10.4 ± 0.6* | 66 ± 8* |
| Ringers and Indomethacin | 33 ± 2 | 12.4 ± 1.0 | 8.9 ± 0.6* | 152 ± 16* |

*significant at $P < 0.01$

The data indicates that renal arterial infusion of indomethacin increases the renal excretion of sodium. An increase in sodium excretion without an appreciable change in the delivery of tubular fluid out of the proximal tubular and with a decrease in $C_{H_2O}$ suggests that this effect of indomethacin on tubular sodium reabsorption is located in the distal nephron. The decrease in urinary prostaglandin- E like material from 2.48 ng/min to 0.8 ng/min with indomethacin suggests that these changes in sodium handling by the tubule could be attributed to a decrease in intrarenal prostaglandins. These results suggest that prostaglandins generated within the kidney stimulate tubular sodium reabsorption rather than inhibit it as is the case when prostaglandins of the E series are infused in the renal artery. Further, the values of urinary immunoreactive prostaglandins during infusion of Ringers were similar to the values during infusion of 2.5 percent dextrose and suggests that prostaglandins inhibition is not an essential element in the natriuretic response to Ringers. The overall

results are therefore consistent with the hypothesis that renal prostaglandins main function as antinatriuretic agents.

Significance to Biomedical Research and Institute Program

Prostaglandins appear to exert potent effects on renal function particularly as regards to sodium excretion by the kidney. They could have major importance in normal renal function and contribute to altered renal handling of sodium which is a central feature in many cardiovascular diseases.

Proposed course of Projects: To continue to explore the physiology of intrarenal prostaglandins in renal physiology and to determine the role of prostaglandins in disordered renal function.

Keyword Descriptors:

prostaglandins, renal sodium excretion, indomethacin

Publications:

None.

Project No. Z01 HL 01810-01
1. Hypertension-Endocrine Branch
2. Steroid & Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
July 1, 1974 through June 30, 1975

Project Title: Spironolactone on plasma renin and dog renal histology.

Previous Serial Number:

Principal Investigators: Taylor, A. A., M.D.

Other Investigators: Bartter, F. C. M.D.
Mitchell, J. R., M.D., Ph.D.

Project Description:

Spironolactone, a potassium-sparing diuretic is known to elevate peripheral plasma renin activity (PRA) in human beings and experimental animals (Vaughn, et al., Amer. J. Cardiol. 32: 523, 1973) presumably by depletion of extracellular fluid volume. Sodium depletion by thiazide or mercurial diuretics or dietary sodium restriction increases both peripheral PRA and granularity of the juxtaglomerular (JG) cells in the afferent arteriole of the kidney in animals and man (Hartroft, in Endo. Path., Bloodworth, ed. Williams and Wilkins, 1968, p. 641.). The effects of spironolactone administration on JG cell histology have not been documented previously. Many patients with the syndrome of juxtaglomerular cell hyperplasia are treated with spironolactone because of profound hypokalemia. One of the necessary criteria for the diagnosis of JG cell hyperplasia is histologic confirmation of such hyperplasia. In another group of patients with low renin essential hypertension, Lowder and Liddle (NEJM 291: 1243, 1974) have reported that PRA, increased by spironolactone therapy remained elevated above pre-treatment values for as long as 36 weeks following cessation of spironolactone therapy.

Objectives: 1) To compare spironolactone- induced changes in plasma renin activity with changes in renal histopathology in dogs, both during drug treatment and at regular intervals following cessation of treatment.

Methods Employed: 1) Dogs are housed in metabolic cages, fed a diet of known sodium content for 1 week per month and urine collected daily. 2) After one week of fixed dietary sodium intake, blood is drawn for the measurement of plasma renin activity (measured by radioimmunoassay of generated angiotensin I), sodium, potassium, blood urea nitrogen, creatinine aldosterone and cortisol (measured by radioimmunoassay). On an aliquot of each daily urine sample are measured sodium, potassium and aldosterone (measured as pH, labile aldosterone by

radioimmunoassay). 3) The dog is then anesthetized with pentobarbital and a small piece of one kidney is removed by retroperitoneal approach using aseptic techniques. 4) For the next 3 weeks each dog is allowed ad libitum food and water and is given a single daily dose of spironolactone, 200 mg. The metabolic balance study and open renal biopsy is then repeated. 5) Each piece of renal tissue is divided; one-half is processed and stained with hemataxylin and eosin, the other half is processed and stained with Bowie's stain. The identification of each slide is masked; an estimation of JG hyperplasia is made on the Hematoxyline-eosin sections and an index of JG cell granularity determined on the Bowie stained sections by a previously described method (Hartroft in Endo. Path., Bloodworth, ed., Williams and Wilkins, 1968, p. 641).

After one month of spironolactone (200 mg/day) treatment of 6 dogs, plasma renin activity has increased from 0.99 ± 0.31 ng/ml/hr (mean ± SEM) (pretreatment) to 4.92 ± 1.04 ng/ml/hr. After 2 months of drug treatment, the mean value in 3 dogs is 5.05 + 1.37 ng/ml/hr. Urinary aldosterone excretion ( measured daily for the last 3 days of each balance period) was 0.074 + 0.022 ug/24 hrs. (n = 6) in the pretreatment period and was 0.585 ± 0.070 ug/ 24hrs. after one month of therapy. No significant changes in plasma, BUN, creatinine, sodium or potassium had occurred after one month of therapy in 6 dogs and after 2 months of therapy in 3 dogs. The renal histology has been reviewed and a JGI determined for each slide but the code will not be broken until the study is completed.

Keyword Descriptors:

plasma renin activity (PRA), spironolactone

Honors and Awards:

Publications:

Project No. Z01 HL 01811-01
1. Hypertension-Endocrine Branch
2. Steroid & Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Metabolism of albumin on patients with idiopathic edema.

Principal Investigators: Dominguez, M., M.D.
Gill, Jr., J. R., M.D.

Other Investigators:

Cooperating Units:

Project Description:

Our previous work has shown that total circulating pool of albumin (TCA) is significantly less than in normal women. The low value for TCA was associated with an increase in fractional catabolic rate per day of albumin and occasionally with a decreased rate of synthesis of albumin. The presence or absence of hypoalbuminemia is being correlated with the morning to evening weight gain as outpatient.

Patients are evaluated as regards to thyroid, liver or kidney functions. When sodium excretion is stable on a sodium take of 109 mEq per day, 5 uCi of $^{131}I$ Serum albumin is given intravenously and aliquots of blood and urine are collected for seven days.

a) Plasma volume: Injected doses in cp 10 minutes/serum cp 10'-BKG.
b) Plasma volume ml/kg= Plasma volume/TBW
c) Total circulating albumin= P.V. ml/kg X albumin concentration.
d) Fractional Catabolic Rate is calculated as the total of counts in the urine divided by the plasma counts divided by plasma volume.
e) Synthetic rate is obtained as TCA X FCR.

| Results | Numbers | TCA gm/kg | FCR | SR gm/kg/day |
|---|---|---|---|---|
| 1. Normal women mean | 10 | 1.61 | .11 | .18 |
| 2. Idiopathic edema | | | | |
| | a) normal 3 | 1.58 | .108 | .17 |
| | b) hyper-catabolic hypo-albuminemia 2 | 1.25 | .144 | .18 |
| | c) hypospynthetic hypo-albuminemia 1 | 1.37 | .096 | .132 |

Three of six patients with a complaint of fluids retention have a decrease in TCA. The cause of the decrease was an increase in catabolism of albumin in two and a decrease in synthesis in one. One of the patient with normal albumin metabolism had hypoplasia of the lymphatics in both legs. The basis for the complaint of edema in the other two is uncertain but tabulation of morning and evening weights indicate little postural weight gain (2 lbs. or less) which is the hallmark of the disorder.

Keyword Descriptors:

idiopathic edema, albumin, hypoalbuminemia

Honors and Awards:

Publications:

Project No. Z01 HL 01812-01
1. Hypertension-Endocrine Branch
2. Steroid & Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Coronary artery disease and urinary steroids

Previous Serial Number:

Principal Investigator: Licata, A. A., M.D., Ph.D.

Other Investigators: Vestergaard, P., M.D.
Bartter, F. C., M.D.

Project Description:

It has been reported that patients with myocardial infarctions excrete elevated urinary levels of 11-ketoetiocholanolone, 11-B-hydroxy-androsterone, 11-B-hydroxyetiocholanolone. This project attempts to verify these findings and to determine if patients with coronary artery disease, but without myocardial infarct, also excrete increased amounts of steroid in comparison to a control group. Thirty patients have volunteered for the project and comprise one of three groups-normals, coronary artery disease without infarct, coronary artery disease with infarct. Preliminary data from two patients in each group have been collected. These data are being analyzed to determine the optimum number of urine specimens needed to insure statistical validity for the remainder of the groups.

Keyword Descriptors:

Coronary artery disease; steroids

Honors and Awards:

Publications:

Project No. Z01 HL 01821-01
1. Hypertension-Endocrine Branch
2. Steroid & Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Nephrogenous cyclic AMP as a parathyroid function test.

Previous Serial Number: None.

Principal Investigator: Broadus, A.E., M.D., Ph.D.

Other Investigators: Bartter, F.C., M.D.

Cooperating Units: Mahaffey, J.E., M.D. and Neer, R.M., M.D., Endocrine Unit, Massachusetts General Hospital, Boston, Mass.

Project Description:

The central objective of this research has been to demonstrate that the measurement of nephrogenous cyclic AMP is a valid index of parathyroid function. The physiologic rationale of this analysis was provided in detail in a previous Project Report (1973-74) and will not be reviewed here.

Initially, we examined the diagnostic utility of nephrogenous cyclic AMP in a pilot series of approximately 10 hyperparathyroid patients and several chronically hypoparathyroid patients. The results were sufficiently encouraging that our objective has changed to the collection of a large clinical series designed to firmly establish the usefulness of nephrogenous cyclic AMP in parathyroid disease (see Major Findings, below).

During the course of these investigations it has become apparent that nephrogenous cylcic AMP might be uniquely useful in studying normal parathyroid physiology and subtle errors in calcium metabolism (see below). Therefore, in the past 4 months an objective of our work has been the study of patients with hypercalciuria as well as normal subjects under a variety of circumstances.

Methods Employed:

Plasma and urinary cyclic AMP are measured by radioligand and assay after column chromatography. Plasma immunoreactive PTH is measured by a well-characterized antiserum (GP-1). Other analyses

are routine.

Standard calcium infusions (4 mg/kg/hour) are performed in selected mild cases of hyperparathyroidism and in occasional hypercalciuric patients, with determinations of nephrogenous cyclic AMP and PTH.

Major Findings:

Data is presently available from 10 patients with hypoparathyroidism, 35 control subjects and 39 cases of hyperparathyroidism. The completed series will contain 10, 50 and 45-50 patients in the 3 groups, respectively. In addition, approximately 8 patients with non-parathyroid hypercalcemia have been studied. The clearance ratio of cyclic AMP to creatinine is the simplest expression of nephrogenous cyclic AMP, values progressively greater than unity indicating increasing renal contributions of the nucleotide. The results ± SEM are:

| Patients (n) | Serum Ca (mg/dl) | PTH# (uleq/ml) | CcAMP/Ccr | Urinary cAMP (nmole/min) |
|---|---|---|---|---|
| HypoPT (10) | 7.9 ± 0.3 | 34 ± 2** | 1.23 ± 0.03 | 1.91 ± 0.15 |
| Normal (35) | 9.5± 0.1 | 59 ± 3 | 1.81 ± 0.12 | 2.82 ± 0.21 |
| HyperPT (39) | 11.4 ± 0.2 | 156 ± 28 | 3.64 ± 0.13 | 4.80 ± 0.34 |
| HyperPT, mild (14) | 10.5 ± 0.1 | 93 ± 6 | 3.42 ± 0.11 | 4.43 ± 0.37 |
| HyperPT, azotemic (8) | 12.1 ± 0.6 | 299 ± 76 | 3.71 ± 0.24 | 3.03 ± 0.57 |

#normal range PTH 30-80 uleq/ml
**undetectable in 5 patients

In the table above, the hyperparathyroid group is subdivided into 14 patients with mild disease (serum calcium 10.8 mg/dl) and 8 patients with moderate renal failure (creatinine clearance 39 ± 3 ml/min). In all cases, the clearance ratio was greater than 2.8; PTH was in the normal range in about 20% of cases. From this series we have concluded that nephrogenous cyclic AMP is a sensitive, reliable and useful tool for study of the spectrum of parathyroid disease.

Although urinary cyclic AMP is, on the average, derived in roughly equal parts from the circulation (by glomerular filtration) and from the nephrogenous pool, we have found that plasma cyclic AMP varies over only a 2 1/2 to 3 - fold range in spite of filtration rates which vary over a 10-fold range (15-150 ml/min). This suggested that correlation of cyclic AMP excretion with simultaneously determined creatinine clearance (nmoles cyclic AMP per 100 ml GFR) might provide a strikingly simple test (plasma cyclic AMP analysis is not required), which would be of wide availability and utility. Our data indicates that this simple determination is extremely useful and will likely become the preferred analysis and means of data expression in the near future.

Patients with mild hyperparathyroidism may offer extreme difficulties in differential diagnosis, in large measure because of their tendency to present with only infrequent episodes of frank hypercalcemia. In 5 such patients, we have found that a standard calcium infusion fails to suppress nephrogenous cyclic AMP normally. PTH analyses during these infusions were difficult, or impossible, to interpret (as noted by others).

There is much controversy concerning the pathogenesis and treatment of idiopathic hypercalciuria, owing largely to conflicting and inadequate methology in the study of such patients. Although our findings in these patients are preliminary, it appears that nephrogenous cyclic AMP offers the most sensitive approach to the study of this disorder. There investigations will form a major part of our objective during the coming year.

Keyword Descriptors:

parathyroid, cyclic AMP

Honors and Awards:

Publications:

Two manuscripts are currently in preparation.

Project No. Z01 HL 01822-01
1. Hypertension-Endocrine Branch
2. Steroid & Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Aminoglycoside effects on urinary calcium.

Previous Serial Number:

Principal Investigator: Licata, A., M.D., Ph.D.; McMurtry, R., M.D., Ph.D.

Other Investigators: Bartter, F.C., M.D.

Cooperating Units:

Project Description:

Preliminary in vivo data from studies with rats showed that acute dose of aminoglycosides altered urinary calcium clearance. We investigated this possibility in normal volunteers by placing them on a constant calcium diet and then monitoring their urinary ion of electrolytes after a 24-hour dose of gentamycin and kanamycin. Preliminary results suggest that these drugs caused a decrease in urinary calcium and sodium.

| | Gentamycin | | | Kanamycin | | |
|---|---|---|---|---|---|---|
| Day | Na | K | Ca | Na | K | Ca |
| 1 | 120+ | 58 | 267 | 171 | 84 | 143 |
| 2 | 99 | 60 | 236 | 93 | 74 | 136 |
| 3 | 58 | 70 | 199 | 67 | 70 | 141 |
| *4 | 47 | 57 | 194 | 90 | 69 | 140 |
| 5 | 44 | 63 | 163 | 52 | 71 | 94 |
| 6 | 48 | 62 | 169 | 67 | 68 | 110 |

*drug given

+values are mean results from two volunteers

Keyword Descriptors:

aminoglycosides, renal electrolytes

Honors and Awards:

Publications:

Project No. Z01 HL 01823-01
1. Hypertension-Endocrine Branch
2. Steroid & Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Vitamin D metabolism in renal stone disease.

Previous Serial Number:

Principal Investigator: Licata, A., M.D., Ph.D.; Bartter, F.C., M.D.

Other Investigators:

Cooperating Units:

Project Description:

A number of clinic patients with nephrolithiasis are being seen, some of whom have hyperabsorption, and others idiopathic hypercalciuria. We propose to look at the vitamin D status of these two classes of patients by developing a suitable vitamin D assay. This project consists of the establishment of a 25-hydroxycholecalciferol receptor assay and its subsequent use in screening these patient populations. Although the development of the assay is underway there are no data to report at this time.

Keyword Descriptors:

vitamin D status in nephrolithiasis

Honors and Awards:

Publications:

Project No. Z01 HL 01824-01
1. Hypertension-Endocrine Branch
2. Steroid & Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Chemical regulators of parathyroid gland secretion.

Previous Serial Number:

Principal Investigator: Licata, A., M.D., Ph.D.; Bartter, F.C., M.D.

Other Investigators:

Cooperating Units:

Project Description:

Recent scientific literature has suggested the possibility that the secretion of parathyroid hormone can be controlled by certain vitamin D metabolites. This implies that therapeutically it may be feasible for specifically designed drugs to modify disease of the parathyroid glands. A preliminary step in this direction is the establishment of an in vitro culture system to test the effect of specific drugs on parathyroid gland function. This has been commenced. No data available yet.

Keyword Descriptors:

parathyroid gland metabolism in vitro

Honors and Awards:

Publications:

None

Project No. Z01 HL 01825-01
1. Hypertension-Endocrine Branch
2. Steroid & Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Evaluation of sodium cellulose phosphate (S.C.P.)

Previous Serial Number:

Principal Investigator: Dominguez, M., M.D.

Other Investigators: Licata, A. A., M.D., Ph.D.

Cooperating Units:

Project Description:

To determine the efficacy of S.C.P. in a long-term study (one year), of patients with hypercalciuria secondary to intestinal hyperabsorption of calcium.

The patients were characterized in the basis of 24 hour urinary calcium over 200 mg, on a calcium diet of 600 mg. Normal or low PTH serum concentration. Low cAMP urinary excretion and high calcium absorption after the administration of 47-Ca.

Nine patients were identified with this disorder and started on S.C.P. 5 gm three times a day. Every four months they have been evaluated as outpatient and at the end of one year treated as inpatient.

The evaluations included some determinations in blood such as Ca, P, Mg, alkaline phosphatase, HS, Ht., WBC, liver function test, creatinine clearance, cooper, iron, zinc, PTH and in urine Ca, P, Mg, Na, K, creatinine, cAMP, and zinc. A plain film of abdomen was taken at the same time, too.

Major Findings: Good tolerance to S.C.P., no evidence of new stones were found during this period, and no side effects in relation with the treatment.

The study will be completed in August, 1975.

Keyword Descriptors:

sodium cellulose phosphate, hypercalciuria, nephrolithiasis

Honors and Awards:

Publcations:

Pak, C.Y.C., Delea, C.S., and Bartter F.C. Treatment of recurrent nephrolithiasis with cellulose phosphate. New England J. Med. 290: 175-187, 1974.

Project No. Z01 HL 01841-01 HE
1. Hypertension-Endocrine Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Binding and Metabolic Effects of Neurotoxic Drugs

Previous Serial Number: None

Principal Investigator: Donald F. Bogdanski, Ph.D.

Other Investigators: Walter Lovenberg, Ph.D.
Hans G. Baumgarten, M.D.

Cooperating Units: None

Project Description:

Objectives: Neurotoxic drugs are currently in wide use in pharmacological laboratories because of their ability to destroy specific cells in the central nervous system. The cells destroyed belong to specific cell groups having a particular neurotransmitter. At present, the mechanism of action of the neurotoxic drugs is unknown. Evidence reported from this laboratory suggests that specificity is a matter of uptake by the cell. The drugs may be generally toxic to all cells provided they penetrate to the interior. The purpose of the present investigations was to determine the mechanism of toxicity and describe it at a molecular level if possible. It was thought that the toxicity might be related to the formation of quinones. If so, the site of a generalized toxicity might be mitochondria, in which the transport of electrons from NADH is inhibited by certain quinones, such as rotenone. This idea served as the starting point for the experiments to be described.

Materials and Methods: Radioactive $C^{14}$-labeled 5,6-dihydroxytryptamine and 5,7-dihydroxytryptamine, and $C^{14}$-parachloroamphetamine were synthesized and supplied by Dr. K. Schlossberger of the Max Planck Institute, Germany.

The subcellular distribution of labeled drugs in rat brain was investigated by density gradient centrifugation. The 12,000 x g ($P_2$) pellet was resuspended and layered over a discontinuous density gradient consisting of 1.4, 1.2, 1.0, 0.8 M sucrose and the $P_2$ suspension in 0.32 M sucrose. After 90 minutes centrifugation at 56,000 x g, the particles fractionate at the various interphases, from low to high density, as follows: A, myelin; B, membranes and small synaptosomes; C, synaptosomes and small mitochondria; D, mitochondria and large synaptosomes; E, nonspecific debris.

The gradient is drained into counting vials, 5 drops per vial, and the radioactivity in each vial determined by scintillation counting.

Major Findings: The different drugs used thus far can be classified according to their binding in specific cellular subfractions. Three distinct classifications were apparent: 1) both 5,6- and 5,7-dihydroxytryptamine were localized in the mitochondrial fraction. Moreover, 5,7-dihydroxytryptamine was apparently covalently bound as determined by exhaustive extraction with ether and methanol. Because of the tenacious binding, these compounds remained in the mitochondrial fraction for at least 24 hours, at which time the radioactivity in the other fractions had fallen to low levels. 2) Colchicine was present in higher concentrations in the synaptosomal fraction than in other fractions. The rate of disappearance at 1, 4, and 24 hours was proportional in all fractions. 3) After 24 hours para-chloroamphetamine was bound to the membrane and the myelin fraction.

In contrast, 5-hydroxytryptamine disappeared from all fractions within 24 hours. Thus, all neurotoxic drugs are tightly bound to specific cell fractions in brain tissue.

The data from experiments with the dihydroxytryptamines, together with earlier morphological data, suggest a mitochondrial site of action for these drugs. In preliminary experiments 5,7-dihydroxytryptamine and 6-hydroxydopamine inhibited reduction of cytochrome b in the presence of NADH and cyanide in rat liver and bovine adrenal medullary mitochondria.

Significance to Biomedical Research and Institute Program: Interest in these compounds is related to their ability to destroy specific groups of cells in the brain. For example, the tryptamines can eliminate serotonergic cells whereas 6-hydroxydopamine can eliminate adrenergic neurons. The specificity of neurotoxicity can thus be used to study the functions of a specific group of neurons in the central nervous system.

The mechanism of toxicity is currently the object of a lively controversy in biomedical research. The investigations reported here suggest that the dihydroxytryptamines destroy cells directly by their mitochondrial toxicity. The manufacture of vital energy resources, or other mitochondrial functions, might be blocked. This would cause the death of the cell. Specificity of cell toxicity is determined by the entrance of the compound into the cell. Serotonergic cells transport these compounds, hence they are destroyed.

Proposed Course of Project: The main objective of future research is to extend observations on mitochondrial toxicity. The inhibitory effect of neurotoxic drugs on electron transport, respiration, oxidative phosphorylation, lipid metabolism and protein metabolism will be studied. The possible usefulness of the dihydroxytryptamines in carcinoid tumors should be considered.

Keyword Descriptors: neurotoxicity 5,6-dihydroxytryptamine 5,7-dihydroxytryptamine synaptosomes mitochondria

Honors and Awards: None

Publications: None

Project No. Z01 HL 01842-01 HE
1. Hypertension-Endocrine Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Electron Transport in Bovine Adrenal Medullary Vesicles

Previous Serial Number: None

Principal Investigator: Donald F. Bogdanski, Ph.D.

Other Investigators: John W. Greenawalt, Ph.D.
Glen Decker

Cooperating Units: Johns Hopkins Medical School
Department of Physiological Chemistry
Baltimore, Maryland

Project Description:

Objectives: This work was based on previous investigations of metabolically dependent retention of norepinephrine by adrenergic neurons in rat heart slices. Retention may be more complex than usually described, that is, a simple binding of amine with ATP, divalent cation and protein. Moreover, extravesicular $Mg^{++}$-ATP is probably not sufficient to account for vesicular retention of NE as proposed by the Scandinavian School of Neurochemists. Their conclusion was based upon studies with isolated bovine adrenal medullary or splenic nerve vesicles. The isolated nerve vesicles do not retain NE at 37° even in the presence of Mg-ATP. Moreover, our investigations revealed metabolic dependencies that could not be anticipated from the results of studies with isolated vesicles. Thus, retention was studied in auto-dialyzed heart slices in which the plasma membrane is the effective dialysis membrane. During auto-dialysis, endogenous substrates are removed from, and exogenous substrates together with metabolic inhibitors, were added to the cytosol. The vesicles are metabolically isolated and controlled in their in vivo location within the neuron.

Retention is dependent upon intermediary metabolism and respiration, but is independent of oxidative phosphorylation. In the absence of glycolysis, inhibitors which eliminate oxidative phosphorylation may or may not block metabolically dependent retention depending upon the occurrence of electron transport. Thus, electron transport is required for metabolically dependent retention (blocked by antimycin A). Electron transport apparently is the basis for the generation of energized state (dissipated by dinitrophenol) but not oxidative phosphorylation (not blocked by oligomycin or by atractyloside). Based on these studies, a decision was made to study electron transport directly in isolated adrenal medullary vesicle membranes.



SD3

Major Findings: Our studies have added significantly to the findings of Flatmark et al., who proposed a conventional electron transporting chain as follows:

Dehydrogenase → Flavoprotein → Cytochrome $b_{561}$

→ Oxidase (presumed) → Oxygen

Our studies indicate the existence of a new cytochrome in the membranes having an α absorption maximum at 555 to 556 nm and which is distinct from cytochrome $b_5$ which has a similar α absorption band. The evidence for the existence of this cytochrome is as follows: 1) at room temperature, the asymmetric α band is split in the presence of some Krebs cycle intermediates, by low concentration of dithionite or by oxygenation of membranes completely reduced by dithionite. 2) The two absorption maxima show an independent temporal development in the presence of cyanide, or $Ca^{++}$ plus cyanide, and 3) their relative steady state maximum absorption differs at various pH.

A second new finding was the existence of a cyanide sensitive factor absorbing at 444 nm in the presence of cyanide. This factor may be similar to that earlier reported in liver microsomes by Sato and by Gaylor. It also may correspond to the presumed oxidase of Flatmark.

A third new finding was the response of the cytochromes to oxygen and to Krebs cycle intermediates. Of the latter group, the response may be absent (citrate), slight (pyruvate, isocitrate, succinate, fumarate and malate), or moderate (α-ketoglutarate, oxaloacetate). It is not yet known whether these responses represent specific dehydrogenase activity.

The cytochromes are oxidized in the presence of dopamine but not norepinephrine. The cytochromes are reduced by pyridine nucleotides and oxidized by ATP. Generally, responses were not inhibited by antimycin A, rotenone or dinitrophenol but studies are incomplete.

A new electron transport pathway is proposed:

Endogenous reducing equivalents → Cytochrome $b_{561}$ →

Cytochrome $C_{555.5}$ → Cyanide sensitive factor → $O_2$

The enzymes discussed here are absent from storage granules in mast cell tumors.

Significance to Biomedical Research and Institute Program: These studies of the storage organelles of the synapse have the same general significance for the biochemistry, physiology and medical applications of synaptic junctions outlined in the report on the efflux of norepinephrine from rat heart slices. The finding of a new enzyme is, of course, extremely significant in biochemistry.

Proposed Course of Project: The relationship between the cyanide sensitive



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factor, oxygen, endogenous reducing equivalents and cytochromes will be established. Evidence for the occurrence of these factors in other storage granules will be sought. The function of these cytochromes and electron transporting factors will be investigated. Obvious roles such as the electron donor for the β hydroxylation of dopamine will be explored. A possible relationship between the electron transporting pathways and metabolically dependent retention, and the metabolism of membrane constituents will be investigated.

Keywords: adrenal medullary vesicles membranes cytochromes electron transport catecholamines cytochrome $b_{561}$ cyanide sensitive factor cytochrome$_{555.5}$

Honors and Awards: None

Publications: None

Project No. Z01 HL 01843-02 HE
1. Hypertension-Endocrine Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Release of Norepinephrine from Neuronal Vesicles

Previous Serial Number: NHLI-37

Principal Investigator: Donald F. Bogdanski, Ph.D.

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: These investigations were designed to study some characteristics of the amine pump located at the plasma membrane of adrenergic nerve endings, and to apply the findings to mechanisms of synaptic transmission, if possible.

Previous publications of work at the Laboratory of Chemical Pharmacology, NHLI, have described some properties of a saturable, $Na^+$-dependent transport mechanism for norepinephrine (NE) in peripheral and isolated brain nerve endings (synaptosomes). A model synapse was devised based upon the $Ca^{++}$-dependent efflux of stored $^3H$-NE from heart ventricle slices incubated in $Na^+$-deficient ($choline^+$) Krebs bicarbonate medium. The efflux was found to be $Na^+$-dependent and inhibitable by desipramine, an inhibitor of the amine pump. Published work has shown that synaptic transmission can be inhibited by desipramine. The present work extends these findings.

Major Findings: The efflux of $Ca^{++}$ mobilized $^3H$-NE from rat heart slices incubated in sodium deficient Krebs-$HCO_3$ ($choline^+$) was studied. Slices were prepared from rats injected with $^3H$-NE 18 hours previously. The slices were incubated 60 minutes in standard Krebs-$HCO_3$ medium in order to re-establish physiological conditions. The slices were then transferred to the $Na^+$-deficient media. Previous work has established that the $^3H$-NE is bound within the endogenous pool after 18 hours. This amine is mobilized by $Ca^{++}$ and appears in the $Na^+$-deficient medium from which 85% of the released radioactivity is recovered as the free base.

The rate of efflux was greatly reduced by 1 to 3 μM desipramine and 1 to 3 μM cocaine. In contrast, phenoxybenzamine at 1 to 100 μM slightly reduced the rate of efflux. The negative result of the phenoxybenzamine experiment is of interest because all three drugs are reported to be potent inhibitors of amine transport.

At this point there are two main considerations for the observed inhibition



SP6

of efflux. The inhibitors act either upon the storage vesicle or upon the amine pump, which, as we have published, may transport intracellular NE to the extracellular medium.

The possibility that the above results are accounted for by a vesicular site of action can be minimized by reports of other laboratories. Thus, cocaine, at 100 μM, produces only 25% inhibition of the efflux of catecholamine from isolated splenic nerve vesicles. This concentration is far greater than the range of concentrations that inhibit efflux. Generally, a disparity in effective concentrations applies to desipramine as well. In contrast, phenoxybenzamine is a potent inhibitor of efflux from isolated vesicles, but had little effect in the above experiments. Thus, the site of inhibition of efflux produced by desipramine and cocaine is the amine pump in the plasma membrane. The weak effect of phenoxybenzamine indicates that this drug does not block the amine pump operating in an outward direction. Since attachment of inhibitor to carrier at the outer surface of the membrane would slow translocation in either direction, it appears likely that phenoxybenzamine has only a weak effect on the transport mechanism. This is in direct contrast to current thinking.

Significance to Biomedical Research and Institute Program: Investigations of the physiological and biochemical characteristics of nerve endings generally are of great interest because synaptic transmission is crucial to either the initiation, control or maintenance (or all three) of all bodily functions, both physical and mental. Synaptic transmission is the point of attack for many drugs currently used for a wide variety of illnesses ranging from hypertension to mental disease. Fundamental knowledge of the nature of the biochemical processes controlling synaptic transmission is crucial to the understanding of the actions of existing drugs, and the development of drugs.

The findings reported above indicate that one aspect of the currently proposed mechanism of the action of one drug was erroneous. Simultaneously, these findings open a door to new knowledge. Thus, why does phenoxybenzamine inhibit uptake of amine if it neither blocks amine transport, nor deplete stores of amine from the nonstimulated nerve like reserpine? Is the answer to this question related to the mechanism by which phenoxybenzamine stimulates overflow of NE from electrically stimulated adrenergic nerves?

The findings reported above together with published reports of inhibition of synaptic transmission by desipramine and apparent inhibition of overflow by cocaine, suggest that synaptic transmission includes a component of outward transport of NE, which can be blocked by transport inhibitors.

Proposed Course of Project: The differences between the effects of desipramine and cocaine, on the one hand, and phenoxybenzamine on the other will be exploited to establish the existence of an outward transport capability in adrenergic nerve endings. Experiments with ouabain and reserpine, together with the drugs will help characterize the amine transport mechanism.

Keyword Descriptors: adrenergic nerve endings storage of norepinephrine outward transport of nerve transmitter desipramine cocaine phenoxybenzamine

Honors and Awards: None

Publications:

Bogdanski, D.F. and Blaszkowski, T.P.: Role of extravesicular adenosine triphosphate and apparent vesicular energy conservation reactions in retention of norepinephrine by adrenergic nerve endings. Neuropharmacology 14: 11-20, 1975.

Project No. Z01 HL 01844-01 HE
1. Hypertension-Endocrine Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Receptors Participating in the Induction of Tyrosine Hydroxylase

Previous Serial Number: None

Principal Investigator: Ingeborg Hanbauer, Ph.D.

Other Investigators: A. Guidotti, M.D.
E. Costa, M.D.

Cooperating Units: Laboratory of Preclinical Pharmacology
National Institute of Mental Health
St. Elizabeth's Hospital
Washington, D.C.

Project Description:

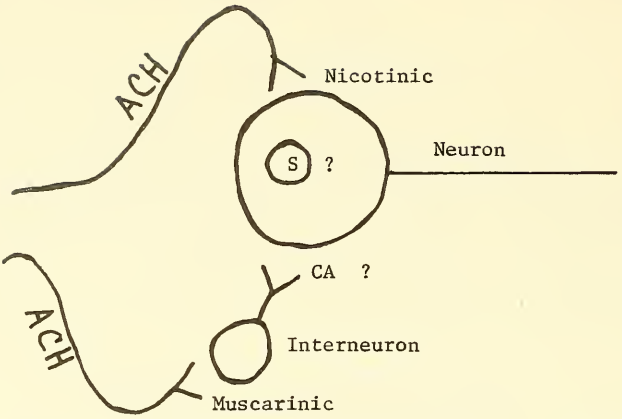
Objectives: In superior cervical ganglia an increased impulse traffic in the preganglionic nerve fibers causes an induction of TH about 48 hours after the stimulation. Decentralization of the superior cervical ganglion or pretreatment with nicotinic receptor blockers curtails this increase in TH activity. This project was carried out to define the role of nicotinic and muscarinic receptors in the induction of TH in intact and decentralized superior cervical ganglia.

Methods: cAMP and cGMP concentration and tyrosine hydroxylase activity were measured as described in the previous report.

Major Findings: Utilizing pharmacological agonists and antagonists specific for various receptors it has been possible to obtain a better understanding on the participation of cholinergic receptors in the regulation of TH induction. Nicotine causes a marked decrease of cGMP content in SCG. Direct activation by nicotine (50 μmol/kg) of nicotinic receptors elicits an increase in TH activity 48 hours after injection. However, a higher dose of nicotine (100 μmol/kg), which presumably also activates muscarinic receptors, fails to induce TH. We therefore studied the effect of methacholine, a muscarinic receptor agonist, and found that it prevented the increase of TH activity. If atropine is used to block muscarinic receptors higher concentrations of nicotine also induce TH. Moreover, atropine facilitates TH induction by nicotine in decentralized superior cervical ganglia. A scheme of the pertinent synaptic connections involved in the regulation of TH induction is shown:



SD9




Reserpine stimulates indirectly the nicotinic receptor. However, in the case of reserpine this interaction by muscarinic receptor agonists is difficult to demonstrate, since reserpine depletes the catecholamines from their storage sites.

Proposed Course of Project: Studies on guanylate cyclase during stimulation of muscarinic and nicotinic receptors will be undertaken to elucidate the molecular mechanisms of these receptor functions. More detailed studies on α- and β-adrenergic receptor agonists and antagonists will be required.

Keyword Descriptors: tyrosine hydroxylase enzyme induction
sympathetic ganglia nicotinic receptors muscarinic receptors

Honors and Awards: None

Publications: None

Project No. Z01 HL 01845-01 HE
1. Hypertension-Endocrine Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Regulation of Tyrosine Hydroxylase by Steroid Receptors
Previous Serial Number: None
Principal Investigator: Ingeborg Hanbauer, Ph.D.

Other Investigators: Walter Lovenberg, Ph.D.
A. Guidotti, M.D.
E. Costa, M.D.

Cooperating Units: Laboratory of Preclinical Pharmacology
National Institute of Mental Health
St. Elizabeth's Hospital
Washington, D.C.

Project Description:

Objectives: It has been reported that enzyme induction in various tissues is under the control of corticosteroids and it is now widely accepted that responses to carbohydrate active steroids are mediated by specific steroid receptor molecules in the cytoplasm. The steroid-receptor complex is then transported to the nucleus, where subsequent interaction with repressor proteins presumably alters transcriptional processes.

Methods: cGMP concentration was measured by $^{32}P$-incorporation into histones catalyzed by a cGMP dependent protein kinase. TH activity was measured by a radiochemical technique with 6-methyltetrahydropterin as cofactor.

Major Findings: Dexamethasone induces TH in sympathetic ganglia, but not in adrenal medulla. This induction is preceded by a sharp decrease in cGMP concentration. Reserpine elicits an increase of plasma corticosterone levels lasting for at least 6 hours. Similarly to dexamethasone, reserpine causes a fall in cGMP content immediately after its administration. The induction of TH elicited by dexamethasone or reserpine was blocked by pretreatment with cortexolone, a metabolite of corticosterone with high affinity for the steroid-receptor protein but without intrinsic glucocorticosteroid activity.

Significance to Biomedical Research and Institute Program: Our results demonstrate that corticosteroids can reduce the cGMP levels in superior cervical ganglion. Considering an antagonistic role of cGMP on cAMP dependent processes in several cellular systems, we can propose an interaction of glucocorticosteroids with the cyclic nucleotide system. This may be important in elucidating mechanisms whereby catecholaminergic cells change their macromolecular composition to adapt to persistent environmental changes.



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Proposed Course of Project: We intend to elucidate: 1) the role of carbohydrate active steroids on the guanylate cyclase system, 2) the role of cGMP in the regulation of the phosphorylated state of non-histone nuclear proteins.

Keyword Descriptors: tyrosine hydroxylase cortexalone corticosterone

Honors and Awards: None

Publications:

1. Hanbauer, I., Guidotti, A., and Costa, E.: Dexamethasone induces tyrosine hydroxylase in sympathetic ganglia but not in adrenal medulla. Brain Res. 85: 527-531, 1975.

2. Hanbauer, I., Lovenberg, W., Guidotti, A., and Costa, E.: Role of cholinergic and glucocorticosteroid receptors in the tyrosine hydroxylase induction elicited by reserpine in superior cervical ganglion. Brain Res., in press.

Project No. Z01 HL 01846-01 HE
1. Hypertension-Endocrine Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Regulation of Tyrosine Hydroxylase in Carotid Body

Previous Serial Number: None

Principal Investigator: Ingeborg Hanbauer, Ph.D.

Other Investigators: Walter Lovenberg, Ph.D.

Cooperating Units: None

Project Description:

Objectives: The carotid body senses physiological oscillations of arterial $pO_2$, $pCO_2$ and $[H^+]$. Fluorescence histochemistry has shown that the carotid body contains catecholamines, mainly dopamine, and indolealkylamines. The transmitters are located in vesicles in the type I cells. It appears that the chemoreception for $pO_2$, $pCO_2$ and $[H^+]$ is carried out by three distinct receptor mechanisms. Our strategy to obtain information on specific transmitters involved in the various types of chemoreception was to study compensatory mechanisms by specific rate limiting enzymes for the biosynthesis of each neurotransmitter.

Methods: Rats are kept in a sealed chamber with a defined volume of atmosphere selected as appropriate to elicit severe hypoxia and hypercapnia in about 30 minutes. Tyrosine hydroxylase activity was measured using a modification of the method described by Waymire et al. (Analyt. Biochem. 43:588-600, 1971) with 6-methyltetrahydropterin as cofactor.

Major Findings: When rats are kept in hypoxic conditions tyrosine hydroxylase activity is increased 20 hours later reaching about twice the values of control rats. If rats are pretreated with apomorphine (5 mg/kg i.p.), a dopamine-receptor agonist, the increase in tyrosine hydroxylase activity elicited by hypoxia and hypercapnia is completely abolished. However, the increase in tyrosine hydroxylase activity is not abated by pretreatment of rats with β-receptor agonists, cholinergic receptor agonists or by specific antagonists for dopamine, norepinephrine or acetylcholine receptors.

Significance to Biomedical Research and Institute Program: From these studies the working hypothesis can be adopted that dopamine is involved in the function of chemoreceptors, which are stimulated by either hypoxia or hypercapnia, or both simultaneously. Since tyrosine hydroxylase is the rate limiting enzyme in the biosynthesis of dopamine the regulation of its induction or activation

must be controlled by hypoxia and hypercapnia.

Proposed Course of Project: The following experimental approaches will be undertaken: 1) Further examination of nature of the increase in tyrosine hydroxylase activity. By an immunoprecipitation technique it will be established whether new synthesis of tyrosine hydroxylase molecules is elicited by hypoxia or hypercapnia. The involvement of second messengers in tyrosine hydroxylase induction which may be activating specific protein kinases will be investigated.

2) Studies on the role of type II cells in the regulation of chemoreceptors with the possible involvement of specific amino acids such as GABA, glycine and taurine will be done.

3) The responsiveness of chemoreceptors to hypoxia in carotid body of spontaneous hypertensive rats (SHR) will be studied.

Keyword Descriptors: carotid body hypoxia tyrosine hydroxylase dopamine

Honors and Awards: None

Publications: None

Project No. Z01 HL 01847-01 HE
1. Hypertension-Endocrine Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Role of Second Messengers in Tyrosine Hydroxylase Induction

Previous Serial Number: None

Principal Investigator: Ingeborg Hanbauer, Ph.D.

Other Investigators: E. Costa, M.D.
A. Guidotti, M.D.

Cooperating Units: Laboratory of Preclinical Pharmacology
National Institute of Mental Health
St. Elizabeth's Hospital
Washington, D.C.

Project Description:

Objectives: The goal of these studies is to understand the effect of neurotransmitters on the control of tyrosine hydroxylase (TH) induction. In adrenal medulla trans-synaptic induction of TH is elicited by direct or indirect stimulation of nicotinic receptors. This stimulation causes an immediate increase in cAMP content and a delayed induction of TH. We applied this model to studies on the superior cervical ganglion of rats.

Methods: cAMP and cGMP concentrations were estimated by measuring the incorporation of $^{32}P$ into histones using cAMP or cGMP dependent protein kinases. TH was measured by a radiochemical technique with 6-methyltetrahydropterin as cofactor.

Major Findings: When adrenal demedullated rats are exposed to cold the cAMP concentration in SCG is increased 3 to 5 times and this increase lasts for several hours. The increase of cAMP content is accompanied by a decrease in cGMP concentration. Since the increase in cAMP could be prevented by propranolol we studied the effect of various beta receptor agonists, dopamine, norepinephrine, and carbamylcholine. Repeated injections of isoproterenol and a single injection of epinephrine caused a significant, long lasting increase of cAMP associated with a delayed increase of TH activity. Both changes could be blocked by propranolol. In contrast, dopamine and norepinephrine caused a minute and short lasting increase of cAMP which was not followed by a delayed TH induction. Carbamylcholine failed to change cAMP and cGMP concentration and TH activity. Decentralization of the ganglion 48 hours before injection of isoproterenol or epinephrine prevented neither the increase in cAMP nor the induction of TH.

Significance to Biomedical Research and Institute Program: It can be concluded from these studies that TH induction can be elicited by stimulation of beta adrenergic receptors (2 hours). This regulatory mechanism for TH induction obtains physiological importance in adrenal demedullated rats, where adrenal medullary compensation mechanisms are absent.

Proposed Course of Project: We intend to elucidate in superior cervical ganglion the role of cAMP in the regulation of the phosphorylated state of non-histone nuclear proteins. This project will be mainly based on the observation that the phosphorylated state of acidic nuclear proteins play an important role in the regulation of gene expression in eukariotic cells and in the modulation of messenger RNA.

Keyword Descriptors: cyclic AMP cyclic GMP sympathetic ganglia tyrosine hydroxylase

Honors and Awards: None

Publications:

1. Hanbauer, I., Kopin, I.J., Guidotti, A., and Costa, E.: Induction of tyrosine hydroxylase by beta adrenergic receptor agonists in normal and decentralized sympathetic ganglia. Role of c-AMP. J. Pharmacol. Exp. Ther. 193: 95-104, 1975.

2. Hanbauer, I., Guidotti, A., and Costa, E.: Involvement of cyclic nucleotides in the long term induction of tyrosine hydroxylase. In Collegium Internationale Congress du Neuropsychopharmacologicum. Paris, France, Excerpta Medica, in press.

3. Guidotti, A., Hanbauer, I., and Costa, E.: Role of cyclic nucleotides in the induction of tyrosine hydroxylase. In Drummond, Robison and Greengard (Eds.): Advances in Cyclic Nucleotide Research. Raven Press, 1975, Vol. 5.

4. Costa, E., Guidotti, A., and Hanbauer, I.: Do cyclic nucleotides promote the trans-synaptic induction of tyrosine hydroxylase? Life Sci. 14: 1160-1188, 1974.

5. Hanbauer, I. and Guidotti, A.: Cyclic AMP dependent regulation of tyrosine-3-monoxygenase in adrenal medulla. Effect of denervation. Naunyn Schmiedeberg's Arch. Pharmacol. 287: 213-217, 1975.

Project No. Z01 HL 01848-01 HE
1. Hypertension-Endocrine Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: A Radioimmunoassay for Dopamine-β-Hydroxylase

Previous Serial Number: None

Principal Investigator: Pauline Lerner, Ph.D.

Other Investigators: Walter Lovenberg, Ph.D.

Cooperating Units: None

Project Description:

Objectives: Dopamine-beta-hydroxylase (DBH) is the enzyme which catalyzes the final reaction in the biosynthesis of norepinephrine. The determination of DBH levels is of interest both for *in vitro* studies of neurochemical systems and for clinical studies covering a variety of physiological and disease states. Several assays for DBH, which are based on the catalytic activity of the enzyme, are now in use. However, interpretation of results from these assays is made difficult by the presence of endogenous DBH inhibitors. In addition, enzymatic assays do not detect enzymatically inactive DBH, which is present along with the functional enzyme in serum. For these reasons, several workers have turned to radioimmunoassay for DBH. A radioimmunoassay, besides being sensitive and specific, would be unaffected by DBH inhibitors and would detect enzymatically inactive DBH. Our objective is to develop such an assay for use in our laboratory.

Methods: Antiserums have previously been produced in rabbits by injection of pure bovine DBH or highly purified human DBH. For iodination of the antigen, chloramine T and $^{125}I$ were reacted with purified bovine DBH.

The coated tube method of radioimmunoassay has been explored. This method is especially well suited for large antigens, such as DBH (molecular weight 300,000), and has been the only radioimmunoassay method to be reported for DBH. In this method, diluted antiserum is placed in a plastic tube, and the antibodies are allowed to bind directly to the tube. Next, an albumin solution is used to cover up nonspecific protein binding sites on the plastic. After this, a solution containing radioactive DBH (and sometimes also unlabeled DBH) is introduced into the tube, and the DBH is allowed to bind to the antibody which is attached to the tube walls. After unreacted material is washed out, the amount of bound DBH can be determined by counting the empty tube.

Major Findings: Experimental techniques have been developed for performing radioimmunoassay of DBH by the coated tube method. The chloramine T method

of radioiodination has been adapted to use with DBH. DBH can be iodinated to a relatively high specific activity without loss of immunoreactivity of the protein, and the iodinated antigen can be recovered without significant contamination from unreacted iodide. Several experimental parameters for the assay itself, including type of plastic, type and concentration of antiserum, and incubation time, have been investigated and optimized. A method for measuring tritiated protein bound to tubes has been worked out. The radioactive part of the tube is cut off, broken up physically, and counted with a counting fluor in a liquid scintillation counter.

This assay can detect immunoreactive material in normal human serum. Preliminary experiments with human cerebrospinal fluid have not enabled us to detect immunoreactive material in this fluid. The assay is currently being used in experiments on protein biosynthesis by bovine adrenal medullae.

Significance to Biomedical Research and Institute Program: DBH is found, along with epinephrine and norepinephrine, in vesicles in the adrenal medulla and sympathetic and central noradrenergic neurons. During synaptic transmission, the vesicles release their contents by exocytosis. DBH from the sympathetic nervous system eventually appears in serum and several investigators have suggested that serum DBH may be a useful indicator of sympathetic activity. Likewise, DBH from the central nervous system might be found in cerebrospinal fluid. If this is the case, DBH levels in cerebrospinal fluid of patients may give useful information on the biochemistry of normal and diseased mental states. The adrenal medullary DBH is also of great interest because its synthesis can be affected by various pharmacological agents. The radioimmunoassay for DBH, which is sensitive, specific, and unaffected by factors which alter DBH enzymatic activity, promises to be a useful tool in studying these phenomena.

Proposed Course of Project: We plan to produce more antiserum directed against human DBH. We hope to obtain an antiserum which has both high specificity and high avidity for DBH. This would enable us to refine our assay for human DBH and make it more sensitive.

We would also like to increase the sensitivity of the assay for bovine DBH so that we can analyze smaller amounts of tissue. We plan to investigate the use of very small polystyrene beads, rather than tubes, as a means of increasing the sensitivity of the assay.

Keyword Descriptors: dopamine-β-hydroxylase immunoassay cerebrospinal fluid schizophrenia hypertension

Honors and Awards: None

Publications: None

Project No. Z01 HL 01849-01 HE
1. Hypertension-Endocrine Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Synthesis of Dopamine-β-Hydroxylase in a Cell Free System

Previous Serial Number: None

Principal Investigators: Pauline Lerner, Ph.D.
Paul MacDonnell, Ph.D.

Other Investigators: Walter Lovenberg, Ph.D.
Gordon Guroff, Ph.D.

Cooperating Units: Section on Intermediary Metabolism
Laboratory of Biomedical Sciences
National Institute of Child Health and Human Development

Project Description:

Objectives: Dopamine-beta-hydroxylase (DBH) is the enzyme which synthesizes the neurotransmitter noradrenalin from its precursor, dopamine. The adrenal medulla is a rich source of this enzyme. Several studies have shown that the adrenal medulla regulates the rate of synthesis of DBH in response to neuronal factors. Nerve impulses arriving at synapses on the adrenal gland can mediate an increase in synthesis of DBH. The chemical link between activity at the synapse and protein synthesis within the cell is not yet understood. If DBH synthesis can be achieved in a cell free system, it would be possible to systematically investigate the effects of various chemical and pharmacological agents on DBH synthesis. Our objective is to develop a cell free system which can synthesize DBH and to study factors which affect this system.

Methods: Fresh bovine adrenal glands are used to prepare RNA. The polysome fraction is first isolated from the adrenal medullae. RNA is obtained from the polysomes by phenol extraction. RNA which contains polyadenylic acid (poly A) is then separated by use of an oligodeoxythymidylic acid column. The poly A RNA (messenger RNA) is used as the template for RNA synthesis in a cell free system derived from untoasted wheat germ.

The protein products of the cell free synthesis are assayed for DBH by means of a radioimmunoassay. The coated tube method of radioimmunoassay is employed, using an antiserum raised against purified bovine DBH.

Major Findings: Preliminary work was aimed at establishing optimum conditions

for recovery of biosynthetically active RNA from bovine adrenal medullae. Extreme freshness of the adrenal glands was found to be essential for high activity. The poly A containing fraction of RNA from the polysomes proved to be the most active RNA fraction for protein synthesis. This fraction of RNA, isolated from fresh adrenal medullae, routinely stimulates protein synthesis to a level four to seven times as high as the background from the wheat germ system alone.

Immunological evidence indicates that some of the material synthesized in this manner is indeed DBH. It appears that 0.2 to 1 percent of the protein synthesized in vitro from adrenal medullary RNA is DBH. RNA preparations from two tissues which do not synthesize DBH, the salivary gland and the adrenal cortex, have also been used in the cell free protein synthesis system. The radioimmunoassay indicates that no DBH is synthesized with either of these two RNAs.

Significance to Biomedical Research and Institute Program: DBH is necessary for the in vivo synthesis of the catecholamines epinephrine and norepinephrine. The catecholamines are of particular interest because of their role in the regulation of blood pressure. Several workers have suggested a possible relationship between serum DBH and hypertension. Brain DBH is essential for the biosynthesis of norepinephrine, which functions as a neurotransmitter in the central nervous system. There is some evidence for a deficiency in brain norepinephrine and DBH in schizophrenics. In both the central and the peripheral nervous systems DBH may be related to disease states. Our studies are designed to elucidate factors which affect the rate of synthesis of this crucial enzyme.

Proposed Course of Project: We now have immunological evidence that DBH is being synthesized in the cell free system which we use. We plan to collect other evidence, using chromatographic techniques, supporting the identity of this material as DBH. We also plan to compare RNA from membrane bound and nonmembrane bound polysomes for their ability to direct DBH synthesis. Since DBH is a protein destined for export from the cell, it may be synthesized preferentially on membrane bound polysomes.

Another goal is to scale down our assay so that we can work with smaller tissue samples. If possible, we will continue our study with tissues from smaller animals which can be subjected to pharmacological treatments.

Keyword Descriptors: protein synthesis dopamine-β-hydroxylase hypertension schizophrenia

Honors and Awards: None

Publications: None

Project No. Z01 HL 01850-06 HE
1. Hypertension-Endocrine Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Biochemistry of the Spontaneously Hypertensive Rats

Previous Serial Number: NHLI-270

Principal Investigator: Walter Lovenberg, Ph.D.

Other Investigators: Yukio Yamori, M.D.
Akinobu Nagaoka, M.D.
Teruhiro Nakada, M.D.

Cooperating Units: None

Project Description:

Objectives: The strain of rats developed in Kyoto, Japan, that exhibit uniform and severe hypertension have been used as an animal model of human essential hypertension. These spontaneously hypertensive rats (SHR) have been studied by numerous investigators for deviations in various metabolic systems. Previous work in this laboratory has established that there appears to be an inverse relationship between catecholamine metabolism in central nervous system and blood pressure. It was also observed that biochemical differences in various normotensive rat strains were marked and that the only strain of any value as a control strain was that of Kyoto/Wistar colony, the parent strain of the SHR. We also observed that there appeared to be an early increase in the rate of lysine incorporation into vascular non-collagen protein. The objective of the current experiments is to examine some of the factors that may be involved in the early increase in the incorporation of lysine into the non-collagen proteins.

Methods: Male SHR were obtained from the NIH animal colony and control Kyoto/Wistar rats were obtained from Taconic Farms, Inc. The SHR were divided into 4 groups at 6 weeks of age. One group had the splanchnic nerve resected, one group was treated with Hexamethonium (50 mg/kg s.c. twice daily), and a third group received Apresoline (80 mg/liter in the drinking water). A group of SHR and Wistar/Kyoto were nontreated controls. The incorporation of $^{3}H$-lysine into proteins of the heart, thoracic aorta, and the mesenteric artery was measured 2 hours following I.V. injection of 50 μc of $^{3}H$-lysine. In heart, the actomyosin was specifically isolated by standard techniques. In the vessels the non-collagen proteins were isolated following extraction with hot 5% trichloroacetic acid. The rate of incorporation was estimated by determining the specific activity of the protein (dpm/mg) and comparing it with the measured specific activity of the serum lysine. Wistar/Kyoto rats (the parent

strain of SHR) were used as control animals. Collagen and elastin were also isolated.

Major Findings: The experiments were performed at 8 weeks of age after two weeks of treatment and the following data obtained:

| | Blood Pressure mm Hg | Mesenteric Artery Protein Collagen dpm/gm tissue | Non-Collagen dpm/gm tissue |
|---|---|---|---|
| Control SHR | 168 ± 2 | 49.0 ± 4.9 | 13.7 ± 0.2 |
| Apresoline | 136 ± 3 | 52.0 ± 3.0 | 15.1 ± 1.5 |
| Hexamethonium | 137 ± 7 | 47.3 ± 3.8 | 8.2 ± 1.0 |
| Splanchnectomized | 136 ± 3 | 58.8 ± 2.7 | 7.9 ± 0.3 |
| Wistar/Kyoto | 131 ± 2 | 34.8 ± 5.8 | 7.8 ± 0.5 |

Protein synthesis was also measured in the heart and aorta and no significant differences were observed. The synthesis of elastin was similar in all groups.

Significance to Biomedical Research and Institute Program: The current work confirms our earlier findings that incorporation of lysine into non-collagen proteins of the small vasculature is increased in young SHR. The possibility that this increase in protein synthesis is neuronally mediated is suggested by the return to control levels in animals given hexamethonium or denervated. Apresoline, a vasodilator which was effective in controlling blood pressure, did not reduce incorporation. These findings would provide common ground for those investigators who suggest that the primary cause of hypertension is neurogenic and those that suggest a primary abnormality in vessel structure.

Proposed Course of Project: 1) Further investigation on the relationship of nerve activity and vascular protein synthesis are planned.

2) These studies will be extended to the new stroke-prone strain of rat that has just been established at the NIH.

3) The peripheral and central aminergic systems will be reevaluated in the stroke-prone rats with particular attention devoted to the prehypertensive stage.

Keyword Descriptors: hypertension spontaneously hypertensive rats catecholamines sympathetic nerves vascular protein synthesis

Honors and Awards: None

Publications: None

Project No. Z01 HL 01851-01 HE
1. Hypertension-Endocrine Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Regulation of Catecholamine Synthesis

Previous Serial Number: None

Principal Investigator: Walter Lovenberg, Ph.D.

Other Investigators: Ingeborg Hanbauer, Ph.D.
Eleanor A. Bruckwick, B.S.

Cooperating Units: None

Project Description:

Objectives: The synthesis of catecholamines in the central nervous system is controlled by the activity of tyrosine hydroxylase. Factors which regulate this enzyme therefore may affect the responsiveness of these neuronal systems. It is known that catecholamine synthetic rates in vivo respond to various drugs and stimulants. The objective of this work is to determine the molecular mechanism by which tyrosine hydroxylase can alter its catalytic activity.

Methods: The striatal area of brains from male Sprague-Dawley rats were used as a source of tyrosine hydroxylase. This enzyme was assayed by the standard tritium release assay.

Major Findings: As reported by others the $K_m$ of tyrosine hydroxylase for its cofactor was reduced to about 1/3 the control value following treatment with haloperidol and other neuroleptics. This activation appeared to be due to change in the macromolecule since it was retained following chromatography on Sephadex. Since these drugs are known to be inhibitors of the dopamine sensitive adenylate cyclase in brain, the effect of cyclic AMP and protein phosphorylating conditions on the enzyme in vitro were examined. Tyrosine hydroxylase activity was measured in 40,000 x g supernatant fractions of rat striata solubilized in 0.2% Triton X 100. Addition of ATP, cAMP, $Mg^{++}$, EGTA, NaF, and theophylline in concentrations optimal for protein phosphorylation to the tyrosine hydroxylation reaction mixture reduces the apparent $K_m$ for 6-methyltetrahydropterin (0.5 mM to 0.15 mM). The reduction in cofactor $K_m$ appears to be similar to that seen in vivo following dopamine receptor blockade and may represent an important regulatory mechanism. This change in cofactor $K_m$ is sufficient to account for a 2- to 3-fold increase in tyrosine hydroxylase activity at estimated physiological cofactor levels (0.1 mM). The increase in activity is totally dependent upon ATP and partially dependent upon cAMP and $Mg^{++}$. Neither ATP (0.5 mM), cAMP (0.1 mM), nor $Mg^{++}$ when added alone shows any effect on the kinetic properties of tyrosine hydroxylase. Cyclic GMP does not

replace cAMP in this system. The above experiments suggest but do not provide direct evidence for tyrosine hydroxylase phosphorylation. Immunoradiochemical studies have thus far been unable to show the direct incorporation of $^{32}P$ from ATP-$\gamma$-$^{32}P$. The rapid removal of phosphate by phosphatases, however, can not be exluded.

Significance to Biomedical Research and Institute Program: The importance of the above findings from a physiological point of view is that, since reduced biopterin is in suboptimal concentration in brain tissue, a rapid and substantial change in the catecholamine synthetic rate can occur even when concentrations of catecholamines remain unchanged. If it is assumed that the tyrosine hydroxylating system is in the dephosphorylated configuration in the resting control animal and that the levels of tetrahydrobiopterin and dopamine are approximately 0.1 mM, the system would be operating at less than 1% of its capacity. The enzyme activity measured in the above experiments is over 700 ng/gram of tissue/hour, whereas the values observed for *in vivo* synthesis are about 1 to 2% of this rate. A decrease in the $K_m$ of the cofactor would result in a proportional increase in *in vivo* synthesis. Activation of a protein kinase or inactivation of a protein phosphatase would result in a rapid decrease in the $K_m$ for tetrahydrobiopterin. This may be the phenomenon which occurs when a lesion is placed in the nigro-striatal pathway or when a neuroleptic drug is given. Based on such a molecular mechanism, receptor mediated feedback inhibition could easily be explained.

Proposed Course of Project: Further attempts will be made to determine whether the tyrosine hydroxylase is directly phosphorylated or whether an activator molecule is involved. Experiments will also be directed toward establishing the molecular commonality between the *in vitro* phosphorylating effects and the *in vivo* drug effects.

Keyword Descriptors: tyrosine hydroxylase catecholamines dopamine neuroleptic

Honors and Awards:

Publications:

1. Lovenberg, W. and Bruckwick, E.A.: Mechanisms of receptor mediated regulation of catecholamine synthesis in brain. In: Usdin, E. and Bunney, W.E. (Eds.): Pre and Postsynaptic Receptors, presented at the American College of Neuropsychopharmacology, Puerto Rico, Dec. 1974, Marcel Dekker, 1975, pp. 149-169.

2. Lovenberg, W., Bruckwick, E.A., and Hanbauer, I.: The effect of ATP, cyclic AMP and magnesium on the affinity of rat striatal tyrosine hydroxylase for its cofactors. Proc. Nat. Acad. Sci., in press.

Project No. Z01 HL 01852-04 HE
1. Hypertension-Endocrine Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Regulation of Hydroxyindole Pathway in the Pineal Gland

Previous Serial Numbers: NHLI-269, NHLI-273

Principal Investigator: Walter Lovenberg, Ph.D.

Other Investigators: Jay S. Skyler, M.D.
Ingeborg Hanbauer, Ph.D.
William Merrick, Ph.D.

Cooperating Units: Molecular Hematology Branch, NHLI

Project Description:

Objectives: The hydroxyindole pathway of the pineal gland offers a unique opportunity to examine mammalian enzyme regulation and regulation of protein synthesis. The synthesis of melatonin is controlled by the levels of serotonin-N-acetyltransferase (NAT) which in turn is under neuronal control. Beta receptor stimulation causes a 50-fold increase in NAT activity. Cyclic AMP is the second messenger in this system. The objective of this project is to define at a molecular level the mechanism by which cAMP is eliciting the large increase in NAT activity.

Methods: Protein kinases were isolated by ammonium sulfate fractionation, gel filtration and DEAE cellulose chromatography. Calf thymus pineal chromatin were isolated by standard techniques. Protein kinase activity was determined by the incorporation of $^{32}P$ into protein using $\gamma$-$^{32}P$-ATP and millipore filtration. Binding of cAMP, and actinomycin D were measured by similar techniques. Template capacity was measured using E. coli polymerase and $^{3}H$-UTP. Serotonin-N-acetyltransferase was measured by radio-techniques.

Major Findings: Both rat and bovine pineal glands have substantial amounts of a cAMP-dependent protein kinase. This cAMP-dependent protein kinase was purified to homogeneity and was found to consist of a catalytic and regulatory (cAMP binding) subunit. It was then reasoned that if protein phosphorylation was mediating the changes in NAT then at least one of three processes must occur: 1) stimulation of gene transcription, 2) increase in rate of translation of -RNA by ribosomal protein synthesis, or 3) a direct activation of the apoenzyme by phosphorylation.

Work on characterization of ribosomal protein kinases has continued. Using reticulocyte ribosomes the kinase activity has been examined by DEAE cellulose,

Sephadex, and phosphocellulose chromatography. Multiple peaks of protein kinase activity are seen. Preliminary characterization of these enzymes showed them to have differing dependencies upon cyclic AMP and cyclic GMP for full activity. Some differences in substrate specificity are observed. In addition to the usual substrates histone, protamine, and casein, we also examined reticulocyte fractions that were enriched in regard to each of the protein synthetic initiation factors. One of the peaks of kinase activity eluted from the DEAE cellulose column preferentially used GTP as the phosphate donor. This may be particularly significant in view of the role of GTP in the protein synthetic mechanism.

Significance to Biomedical Research and Institute Program: It can be concluded from these studies that cAMP may exert an affect on NAT by stimulating protein kinase which in turn activates both transcription and translation. Activation of a precursor protein by phosphorylation does not occur. Therefore, the sequence of events by which neuronal impulses can be translated into changes in enzyme can be hypothesized as follows:

Sympathetic nerve → Norepinephrine → Pineal (β Receptor) → Adenyl cyclase

cAMP → Protein kinase → Increased transcription + Translation → Elevated NAT

Proposed Course of Project: The following experimental approaches will be undertaken:

1. Further definition of the role of phosphorylation in initiation of polypeptide synthesis, especially initiation factors per se,

2. Characterization of the various reticulocyte ribosomal kinases and development of a fractionation strategy for purification,

3. Further examination of the products of chromatin phosphorylation particularly the acidic proteins, using pineal chromatin rather than the heterologous cAMP thymus chromatin,

4. Direct measurement of incorporation of amino acid into NAT and *in vivo* experiments with protein synthesis inhibitors.

Keyword Descriptors: pineal gland melatonin serotonin-N-acetyltransferase protein kinase cyclic AMP

Honors and Awards: None

Publications: None

Project No. Z01 HL 01853-12 HE
1. Hypertension-Endocrine Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies on the Properties of Iron-Sulfur Proteins

Previous Serial Number: NHLI-268

Principal Investigator: Walter Lovenberg, Ph.D.

Other Investigators: Larry F. Bennett
Peter DeBrunner

Cooperating Units: Dept. of Chemistry, University of California, San Diego
Dept. of Physics, University of Illinois, Urbana

Project Description:

Objectives: Iron-sulfur proteins constitute a broad class of enzymes and electron carriers that are distinguished by the presence of one or more iron atoms coordinated exclusively by sulfur atoms at the active center. Although these types of redox centers may be considered primative from an evolutionary point of view, they function innumerable key and sophisticated roles in bacteria, plants and animals today. Previous work from this laboratory has described the discovery, isolation, and extensive chemical and physical characterization of these proteins. The current objectives of this project are to further refine physical-chemical knowledge of certain iron-sulfur electron carriers and to search for new roles for iron-sulfur enzymes in mammalian physiology.

Methods: Clostridium pasteurianum was used as a source of the two proteins under consideration. Pure ferredoxin and rubredoxin were isolated from these organisms by techniques devised in this laboratory and given in previous reports.

Major Findings: Little new experimentation was done on this project during the past year, however, collaborative work continued with Drs. DeBrunner and Bennett. Techniques have been developed for preparation of $^{57}Fe$ rubredoxin samples that are indistinguishable from native rubredoxin. Using this better quality $^{57}Fe$ rubredoxin, magnetic Mössbauer spectra have been obtained with large paramagnetic splitting and excellent resolution. Such studies give us valuable information on the molecular environment of the iron atom and the distortion of its ligands. They now can be coordinated with other types of spectroscopic data.

Significance to Biomedical Research and Institute Program: The work described above is part of our continuing program to elucidate the structure and mechanism of iron-sulfur redox centers in proteins. Although such centers have only been recognized for a little over a decade, it is now apparent that they represent one of the most important mechanisms for the transfer of electrons in biology. While the biological importance of rubredoxin is not well understood, this protein from Clostridium pasteurianum is now probably the most completely resolved protein molecule from both a structural and reaction mechanism point of view. As such it has become a model for the study of other proteins, particularly iron-sulfur proteins.

Proposed Course of Project: 1) Complete Mössbauer spectroscopic study of rubredoxin. 2) Establish a new study using detailed electron exchange methods to obtain greater understanding of the redox mechanism. 3) Start a new series of experiments using a new improved laser Raman instrument to probe the atomic properties of the active site iron.

Keyword Descriptors: ferredoxin rubredoxin iron-sulfur proteins Mössbauer spectroscopy

Honors and Awards: None

Publications: None

Project No. Z01 HL 01854-04 HE
1. Hypertension-Endocrine Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Characterization of Human Dopamine-β-Hydroxylase

Previous Serial Number: NHLI-272(c)

Principal Investigator: Robert C. Rosenberg, Ph.D.

Other Investigators: Walter Lovenberg, Ph.D.

Cooperating Units: None

Project Description:

Objectives: The objective of this project is to isolate and characterize dopamine-β-hydroxylase (DBH) from human plasma.

Methods: 1) Purification: Previous work in this laboratory has shown that by using standard isolation techniques highly purified DBH can be obtained from pooled human plasma. These techniques include ammonium sulfate fractionation, binding to and elution from DEAE cellulose columns and gel filtration on agarose columns. Additional purification steps include: affinity chromatography on tyramine-sepharose columns or concanavalin A sepharose columns.

2) Characterization: DBH from bovine adrenals has been isolated and characterized as to its molecular weight, subunit content, amino acid analysis, carbohydrate content, copper content and steady state kinetic properties. Similar characterization can be done with human DBH.

Major Findings: Purification: As previously reported human serum contains up to 10 μg/ml of DBH. Although the purification procedure developed previously yielded a highly purified enzyme (~2000-fold), the recovery was poor and the final product showed significant contamination by three or four other proteins. Attempts at refining the procedure to both increase the recovery of enzymatic activity and to eliminate the contaminating proteins have been pursued. Modification of the conditions used in the ion exchange and gel filtration steps have resulted in substantial increases in the recovery of enzymatic activity from these steps. It has also been found that the conditions previously reported for Con A-sepharose affinity chromatography of DBH cause considerable loss of enzymatic activity. The use of these unfavorable conditions of ionic strength can explain the poor recoveries of DBH activity found in this and other laboratories. Efforts to obtain reasonable quantities of electrophoretically homogenous human plasma DBH are continuing.

Characterization: The use of inhibitors to study the kinetics of the DBH catalyzed conversion of tyramine to octopamine is considerably complicated by the requirement for catalase in reactions where ascorbate serves as the electron donor. Many if not all of the compounds that have been reported to inhibit DBH also inhibit catalase. Thus a reaction system for DBH which does not require catalase would prove invaluable in elucidating the detailed mechanism of DBH activity. Other laboratories have reported that $K_4Fe(CN)_6$ can serve as the electron donor in DBH catalyzed reactions. Our preliminary results indicate that when $Fe(CN)_6^{4-}$ is used as the electron donor substantial DBH activity can be observed even when catalase is not included in the reaction mixture. Thus, use of a DBH assay system with $Fe(CN)_6^{4-}$ serving as the electron donor permits reevaluation of the compounds that have been reported to inhibit or activate DBH. Towards this end we have found that one of the standard assay procedures for measuring DBH in plasma facilitates removal of the $Fe(CN)_6^{3-}$ produced in the assay reaction. $Fe(CN)_6^{3-}$ would otherwise interfere with the spectrophotometric determination of the reaction product. Further preliminary results have shown that azide ion is a true inhibitor of DBH. Spectroscopic study of the azide-DBH complex should provide information about the number and nature of active sites in DBH.

Significance to Biomedical Research and Institute Program: DBH is essential for the function of adrenergic neurons and chromaffin cells since it catalyzes the final step in the synthesis of the neurotransmitter, norepinephrine. DBH has been found within the synaptic and chromaffin granule vesicles, and upon nerve stimulation it is released along with the neurotransmitter by an exocytotic process. Hyperactivity of the sympathetic nervous system has been implicated in the development or maintenance of essential hypertension in man and experimental forms of hypertension in animals. Detailed molecular knowledge of the structure, function and regulation of DBH is important for a complete understanding of the functioning of the adrenergic nervous system in both normal and disease states. There is a large body of work on the in vivo inhibition of DBH by various chelating and thiol compounds, some of which have been used as antihypertensive agents in man. A detailed understanding of the mechanism by which these inhibitors modulate DBH activity can provide valuable information on the functioning of the native enzyme as well as suggestions for potentially useful new antihypertensive agents.

Proposed Course of Project: Work on purifying human DBH from plasma is continuing. Simultaneously, steady state kinetic studies on the effects of both chemical and pharmacological inhibitors and activators of DBH are being pursued using partially purified enzyme. These studies are and will be carried out under conditions where the enzyme activity is not catalase dependent.

Keyword Descriptors: serum dopamine-β-hydroxylase hypertension adrenal glands

Honors and Awards: None

Publications: None

Project No. Z01 HL 01855-01 HE
1. Hypertension-Endocrine Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Catalogue of Isohormones of Human Growth Hormone

Previous Serial Number: None

Principal Investigator: Jay S. Skyler, M.D.

Other Investigators: Andreas C. Chrambach, Ph.D.

Cooperating Units: Reproduction Research Branch, NICHD

Project Description:

Objectives: 1) To develop a catalogue of the various isohormone species of human growth hormone (hGH) in relation to molecular structure.

Methods: The catalogue is based on results obtained by three techniques: 1) quantitative polyacrylamide gel electrophoresis in a multiphasic buffer system optimized for the characterization of hGH; 2) isoelectric focusing in polyacrylamide gel; 3) polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) in the presence and absence of reducing agents. Polyacrylamide gel electrophoresis and SDS-PAGE are responsive to variation in molecular size, while polyacrylamide gel electrophoresis and isoelectric focusing in polyacrylamide gel detect variation in molecular net charge. In polyacrylamide gel electrophoresis, the joint 95% confidence envelope of retardation coefficient (a parameter of molecular size) and relative free mobility (a parameter of molecular net charge in a given buffer milieu), serves as the best single way of identifying and defining isohormone species.

In addition, reactivity in immunologic assay or bioassay is required by definition before any species can be designated an "isohormone".

Major Findings: Standard hGH preparations, from the National Pituitary Agency of the U.S.A. and from AG KABI of Stockholm, exhibit multiple isohormone species. These are both "many" and "few". They are "many" in that any preparation contains 2 to 5 recognizable isohormones. They are "few" in that only the same group of 5 isohormones has been found in all preparations examined. hGH iodinated by a lactoperoxidase procedure showed preservation of gross confirmation by polyacrylamide gel electrophoresis criteria. hGH iodinated by a gentle "monoiodo" chloramine-T procedure showed slight alteration by polyacrylamide gel electrophoresis criteria, despite showing full activity in both radioimmunoassay and radioreceptor assay.

The related hormones, human prolactin and human chorionic somatomammotropin, have been compared with the multiple hGH forms. Several human prolactin and human chorionic somatomammotropin isohormone species, some of which are indistinguishable from some of the hGH isohormones, have been identified.

Significance to Biomedical Research and Institute Program: Growth hormone is important in growth retardation and in acromegaly. In addition, it may play a role in the vascular complications of juvenile diabetes mellitus, and its secretion is markedly decreased in obesity. Furthermore, it may play a role in anabolic processes in general; e.g., post-myocardial infarction, post-operatively, etc. The current attempts to catalogue the multitude of growth hormone species known will allow better insight into structure-function relationships in terms of these various actions of growth hormone.

Proposed Course of Project: 1) Characterization of additional known species of growth hormone, including circulating "big" growth hormone, fatty acid bound growth hormone, synthetically produced growth hormone.

Keyword Descriptors: growth hormone gel electrophoresis isohormones

Honors and Awards: None

Publications: None

Project No. Z01 HL 01856-02 HE
1. Hypertension-Endocrine Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Control of Growth Hormone Secretion

Previous Serial Number: NHLI-274

Principal Investigator: Jay S. Skyler, M.D.

Other Investigators: Hans G. Baumgarten, M.D., Ph.D.
Walter Lovenberg, Ph.D.

Cooperating Units: None

Project Description:

Objectives: The secretion of growth hormone by the adenohypophysis is under the control of hypothalamic peptides, growth-hormone releasing hormone and growth-hormone release-inhibiting hormone (somatostatin). These hypothalamic peptides in turn are under control of dopaminergic and serotonergic neurons in the hypothalamus and/or other brain areas. Recent evidence has shown that serotonergic activity stimulates release of growth hormone and that dopaminergic activity may either stimulate or inhibit growth hormone release, with some species differences.

Serotonin production is regulated by tryptophan hydroxylase and dopamine production by tyrosine hydroxylase. An accompanying report discusses specific inhibitors of these enzymes.

The objective of this project is to define further the hypothalamic regulation of growth hormone secretion by serotonergic and dopaminergic mechanisms.

Methods: Drugs which are specifically cytotoxic for serotonergic and catecholaminergic neurons are injected intraventricularly into newborn or adult rats. Growth of rats is measured by weight change. Growth hormone is measured by standard radioimmunoassay and destruction of neurons are monitored by measuring changes in tryptophan and tyrosine hydroxylase.

Major Findings: A single dose of 5,7-dihydroxytryptamine (5,7-DHT) to a newborn rat causes a reduction of growth. Likewise, intraventricular administration of 75 μg 5,7-DHT to rats of 8 weeks of age causes a reduction in weight gain over the next several weeks. This dose of 5,7-DHT causes a permanent destruction of about 20% of the serotonergic neurons in the hypothalamus. Circulatory basal growth hormone concentrations, however, are unaltered.

Significance to Biomedical Research and Institute Program: Understanding the monoaminergic control of growth hormone secretion may allow better insight into regulation of growth, including such aspects as the mechanism of psychologically induced growth retardation. Likewise, there is potential for development of pharmacologic agents to either enhance growth hormone secretion (in the case of some growth-hormone deficient patients) or depress its secretion (in the case of acromegalic patients or patients with diabetes mellitus).

Growth hormone levels in patients with juvenile diabetes are elevated, and may be related to diabetic complications. The ability to suppress this elevated growth hormone could potentially help diminish these vascular complications of diabetes.

In addition, these studies may promote the fundamental understanding of neuroendocrine regulation, especially of the hypothalamic-hypophyseal axis. Growth hormone levels may also potentially serve as an index of central serotonergic activity.

Proposed Course of Project: The following experimental approaches will be undertaken:

1. further examination of the effects of inhibitors of tryptophan hydroxylase and tyrosine hydroxylase on growth hormone secretion, with specificity increased for specific CNS activity,

2. attempts to overcome the changes with other substances that stimulate or inhibit growth hormone secretion, including acute administration of agonists,

3. attempts to examine secretion of other pituitary hormones to determine specificity of effects for growth hormone secretion, specifically prolactin, thyroid hormones, gonadal hormones.

Keyword Descriptors: neurotoxin serotonin growth hormone acromegaly

Honors and Awards: None

Publications: None

Project No. Z01 HL 01857-02 HE
1. Hypertension-Endocrine Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Growth Hormone Secretion and Structure in Cultured Cells

Previous Serial Number: NHLI-275(c)

Principal Investigator: Jay S. Skyler, M.D.

Other Investigators: Richard A. Knazek, M.D.
Walter Lovenberg, Ph.D.

Cooperating Units: Laboratory of Pathophysiology, NCI

Project Description:

Objectives: 1) To develop a method of production of large quantities of growth hormone and prolactin for therapeutic purposes. 2) To gain insight into control of hormone secretion in tissue culture.

Methods: Tumors taken from patients undergoing hypophysectomy are grown in tissue culture both in monolayer and in artificial capillaries. Growth hormone and prolactin secretion into tissue culture media is monitored by radioimmunoassay. Agents to stimulate or decrease hormone secretion are added to the tissue culture apparatus and secretion monitored.

Growth hormone and prolactin produced by the tumor are compared with their "normal" serum and pituitary counterparts by immunological dilution in radioimmunoassay, dilution in radioreceptor assay, gel filtration, and quantitative polyacrylamide gel electrophoresis.

Major Findings: We have been able to maintain production of human prolactin in artificial capillaries for as long as five months, and obtained a total of 3 mg human prolactin from a single culture unit. A continuous line of rat pituitary cells inoculated into multiple artificial capillary units has produced amounts of growth hormone and prolactin as high as 100 mcg/day/culture unit. Both human growth hormone (hGH) and human prolactin have also been obtained in culture, in excess of 2000 ng/ml. In confirmation of the work of others, we have found hGH secretion is increased significantly in culture by the addition of hydrocortisone to the media. By using single-pass perfusion for human pituitary cells cultured on artificial capillaries, we have found both an eight-fold immediate and a lesser prolonged response of increased human prolactin secretion to TRH.

We have established four criteria which must be met by hormone produced in tissue culture in order to be considered indistinguishable from "standard"

hormone obtained by pituitary extraction: 1) parallel dose-response immunodilution curves in radioimmunoassay, indicating that specific antibody fails to recognize structural differences; 2) parallel dose-response curves in specific radioreceptor assay, "receptodilution", indicating that receptor binding sites do not discriminate between the compared materials; 3) identical partition coefficients on gel filtration; 4) indistinghishable joint 95% confidence envelopes of retardation coefficient and relative free mobility in quantitative polyacrylamide gel electrophoresis in a multiphasic buffer system optimized for characterization of the hormone in question. Tissue culture produced hGH has met these four criteria. Human prolactin from tissue culture has met the three criteria thus far available in our laboratory (all except receptodilution).

Significance to Biomedical Research and Institute Program: The production of large quantities of growth hormone and prolactin would make these available for both investigative and clinical purposes. Prolactin may be important in breast cancer. Growth hormone is important in growth retardation. It may additionally be important in anabolic processes in general; e.g., after myocardial infarction, postoperatively in osteoporosis, etc. Its usefulness in these conditions could be tested if supplies were available.

Proposed Course of Project: 1) Demonstration of biological activity of culture-derived hormone, both in vitro and in vivo,

2) increased scale of production,

3) development of a purification scheme for the culture-produced hormones,

4) demonstration of freedom of contamination with other materials,

5) further use of the artificial capillary system for physiologic and pharmacologic studies.

Keyword Descriptors: growth hormone prolactin pituitary tumors capillary tissue culture

Honors and Awards: None

Publications: None

Project No. Z01 HL 01858-02 HE
1. Hypertension-Endocrine Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Juvenile Diabetes Mellitus

Previous Serial Number: NHLI-276(c)

Principal Investigator: Jay S. Skyler, M.D.

Other Investigators: George J. Ellis, M.D.
Francine L. Levobitz, R.N.
Stanley Slater, M.D.

Cooperating Units: Division of Endocrinology, Department of Medicine, Duke University, Durham, North Carolina; Carolina's Camp for Diabetic Children; Laboratory of Clinical Sciences, NIMH

Project Description:

Objectives: 1) To improve education of health personnel about diabetes mellitus, 2) to improve education of children with diabetes mellitus about their condition, and 3) to better understand juvenile diabetes, especially complications of diabetes.

Methods: Approximately 110 youths with diabetes mellitus each year attend Carolina's Camp for Diabetic Children. Medical and nursing students live in cabins with groups of 8 to 12 children with diabetes to both learn about the condition and help in instructing the children in proper diabetic management.

Evaluation forms are completed by staff to monitor diabetes education of patients. Follow-up questionnaires are completed by children and parents.

Major Findings: 1) Children can learn proper techniques of insulin administration and urine testing, and continue to utilize these techniques for many months after being taught. They tend not to continue to adhere to proper dietary habits.

2) Complications of diabetes may be detected even in young patients if somewhat sophisticated screening (fundus photography, vibratory perception threshold) is used. These seem to correlate with elevated blood lipids and a history of poor diabetic control.

3). Diabetic camp appears to be the single best setting for education of health personnel about diabetes mellitus.

4) Data were gathered concerning the format and teaching programs of 47 camps for children with diabetes in the United States. This data is to serve as a background for developing guidelines for such camps. Two internaticnal workshops have been held for that purpose.

Significance to Biomedical Research and Institute Program: That control of diabetes is related to vascular complications has become increasingly apparent in light of many recent studies. Since in the past (and even today) this concept has been controversial, normalization of blood sugar has not been the goal of management of most patients with diabetes.

The attainment of that goal is difficult to achieve and requires much effort on the part of physician and patient alike. However, neither has been well-educated in the methods needed to achieve that goal. Since camps for children with diabetes provide a format for education of health personnel and patients, they may provide one of the best formats for achieving the goal of normalization of blood sugar.

Vascular complications of diabetes by no means account for all of the morbidity associated with the condition. Indeed the marked increase in incidence and severity of atherosclerotic disease associated with diabetes is equally important. Our findings indicate there may be a correlation between elevated lipids, early signs of vascular complications, and poor control. Thus education in achievement of normalization of sugar may help to decrease the incidence and severity of atherosclerotic disease and of vascular complications of diabetes.

Proposed Course of Project: 1) Develop teaching aids for patients with diabetes.
2) Develop teaching materials to improve education of health personnel about diabetes.

3) Monitor the effectiveness of these teaching materials.

4) Expand studies of incidence of vascular complications in childhood diabetes.

5) Correlate emotional reaction of campers with diabetes and the complexity and success of their treatment regimen.

Keyword Descriptors: diabetes diabetic camps

Honors and Awards: Dr. Skyler was appointed Chairman of the Committee on Camps, American Diabetes Association.

Publications: None

Project No. Z01 HL 01859-04 HE
1. Hypertension-Endocrine Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Characterization of the Active Site(s) of Dopamine-β-Hydroxylase

Previous Serial Number: NHLI-279

Principal Investigator: Gustavus A. Walker, Ph.D.

Other Investigators: Walter Lovenberg, Ph.D.

Cooperating Units: None

Project Description:

Objectives: The objective of this project is to determine the number of active sites on dopamine-β-hydroxylase (DBH) indirectly by first determining the total number of electrons transferred when DBH goes from the physiologically reduced state to the physiologically oxidized state, and secondly, by determining the number of electrons transferred as a unit through potentiometric titrations.

Methods: DBH was isolated by a modification of previously published procedures. The modification utilized agarose gel filtration as the final purification step. The enzyme was demonstrated to be electrophoretically homogeneous with a specific activity 50% greater than the best preparation of DBH yet published.

Dichloroindophenol and potassium ferrocyanide were selected as suitable redox mediators. The oxidized or reduced form of each mediator was determined spectrophotometrically.

Major Findings: A major obstacle in the investigation of the redox potential of DBH is the requirement for relatively large amounts of the purified protein. The reported yields of DBH from previous investigators are low with equally low specific activities. For example, Foldes et al. isolated from 600 grams (wet weight) bovine adrenal medulla less than 5 mg of DBH with a specific activity of less than 2 μmoles of octopamine produced per minute per mg DBH; Wallace and Lovenberg improved the efficiency of yields from a comparable amount of starting medulla to about 10 mg of pure DBH with a specific activity of about 20 μmoles per minute per mg DBH. By substituting two consecutive agarose gel filtration steps for a Sephadex G-200 step and final DEAE gradient, we are able to isolate from 600 grams of adrenal medulla about 18 mg of DBH with a specific activity of 30 μmoles per minute per mg DBH. The amino acid analysis performed on the DBH purified by this technique gave identical results with that performed on DBH purified by the technique of Wallace and Lovenberg.

The temperature at which DBH is stored can also affect the specific activity with time. For example, purified DBH loses about 50% of its specific activity in a week if stored at 4°C in plastic containers but only 15% of the specific activity is lost in a week if DBH is stored in similar containers at -20°C (part of the loss under the latter conditions may be due to the effects of freezing and thawing). Quite obviously choice and length of purification procedures as well as storage time are important considerations in evaluating protein yields and specific activity.

Only preliminary findings have resulted from the use of dichloroindophenol and ferrocyanide as redox mediators with DBH. While both compounds can couple with DBH only reduced dichloroindophenol can significantly donate electrons to oxidized DBH at equimolar concentration of dye to protein. Several thousand-fold excess of ferrocyanide to DBH are required to transfer electrons to DBH from ferrocyanide. This, of course, suggests that the midpoint potential of DBH is closer to dichloroindophenol ($E_o'$ = +218 mv) than to ferrocyanide ($E_o'$ = +360 mv).

Significance to Biomedical Research and Institute Program: Dopamine-β-hydroxylase catalyzes the final step in the synthesis of the neurotransmitter norepinephrine. Upon nerve stimulation DBH together with norepinephrine is released by an exocytotic process from sympathetic nerve endings and adrenal vesicles to appear in the serum of man. The release of DBH and catecholamines is a process intimately involved in the mechanism of nerve transmission of impulses. In order to fully understand the process of nerve impulse transmission and the physiological effects of released DBH a complete elucidation of the molecular properties of this enzyme, especially the active site(s), is necessary.

Proposed Course of Project: Experiments are planned to determine the stoichiometry of electron transfer using 2,6-dichloroindophenol and potassium ferrocyanide as the respective electron donor and acceptor, and monitor the changes in the Cu(II) electron spin resonance signal. Estimates of the midpoint potential of DBH can be made through dye equilibration with DBH by determining the relative percentage of reduced and oxidized forms of each reactant at equilibrium. Values for the midpoint potential of DBH can be more precisely determined by potentiometric titrations. This technique also allows the determination of a number of electrons transferred to DBH as a unit. Samples could be removed to electron spin resonance tubes at various points during the titration to correlate changes in $E_h$ with changes in DBH oxidation.

Another interesting area to be explored is the equivalence of DBH subunits. Although a few investigators have examined this question, subsequent evaluation of their results suggests that they were working with impure preparations of DBH. End group analysis and cyanogen bromide cleavage are the methods of choice to investigate the equivalence of DBH subunits.

Keyword Descriptors: glycoprotein dopamine-β-hydroxylase copper redox potential

Honors and Awards: None

Publications:

Wallace, E.F. and Lovenberg, W.: Studies on the carbohydrate moiety of dopamine β-hydroxylase: Interaction of the enzyme with concanavalin A. Proc. Nat. Acad. Sci. USA 71: 3217-3220, 1974.

Project No. Z01 HL 01860-01 HE
1. Hypertension-Endocrine Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Mitochondrial Monoamine Oxidase

Previous Serial Number: None

Principal Investigator: Margaret Walker, Ph.D.

Other Investigators: Walter Lovenberg, Ph.D.

Cooperating Units: None

Project Description:

Objectives: Mitochondrial monoamine oxidase (MAO) catalyzes the deamination of neurohumoral and vasoactive amines. Inhibitors of this enzyme have been to be effective therapeutic agents in depression and high blood pressure, however, the mechanisms by which MAO inhibitors exert their therapeutic effect on these diverse diseases are not known.

MAO has been found in at least two major forms with different substrate and inhibitor specificities. Whether the two classes of MAO are distinct enzymes or whether the same enzyme exists in different chemical environments (i.e., with different amounts of bound lipid) has not been distinguished. There is evidence that MAO may be an iron-flavoprotein, however, it has not been well characterized.

The purpose of this project is to determine the amount of iron in pure MAO, the nature of its binding, and its functional significance in the major forms of the enzyme. Characterization of the substrate binding sites will also be explored.

Methods: MAO is being purified from beef liver. Initially the procedure of Youdim and Sourkes (Canadian J. Biochem. 44: 1397, 1966) is being used. The iron content will be monitored throughout the purification procedure, and both serotonin and benzylamine deamination will be assayed to determine whether either the A or B form of MAO is being purified preferentially. Additional procedures which will be attempted, include affinity chromatography, and carrying out purification in the presence of iron and/or sulfhydryl reagents to see if activities can be improved.

MAO is assayed for its ability to deaminate serotonin (MAO type A), benzylamine (type B), and kynuramine (A and B) by standard methods. Total iron will be determined using the ferrous iron chelator O-phenanthroline or $\alpha\alpha'$-dipyridyl under reducing conditions. It may be necessary to release iron with sodium mersalyl.

Major Findings: Work on the purification of MAO from beef liver has just begun. The enzyme at this point has a specific activity of 12,000 μmoles kynuramine per mg per hour. This is comparable to the preparations reported from many laboratories, but is much less than the highest specific activity reported. The visible spectrum of the MAO shows peaks at approximately 410 nm and 490 nm typical of flavoproteins. Iron analysis gives a value of 6Fe/150,000 mw. This high value suggests that the MAO may still be contaminated with some mitochondrial iron-sulfur protein.

Significance to Biomedical Research and Institute Program: The relevance of this study to biomedical research lies in the importance of understanding monoaminergic neuronal systems and the role they play in certain types of diseases. It appears that these monoaminergic systems may be involved in the regulation of blood pressure. Work from this laboratory and others strongly implicate a role for central noradrenergic neurons in the development of hypertension. The role of MAO in regulating amounts and availability of neurotransmitter amines is known, however, the fact that many MAO inhibitors are effectvie antihypertensive agents is not well understood. The hypotensive effect of inhibiting an enzyme which inactivates pressor amines appears paradoxidal but may reflect a mechanism of the central nervous system. Examining MAO at the molecular level may provide insight as to the factors that determine the substrate and inhibitor specificity of the different forms of this enzyme. This knowledge will certainly be of value in designing new and better inhibitors of MAO to be used as antihypertensive agents.

Proposed Course of Project: Work is continuing on the purification of MAO. If highly purified MAO still contains appreciable amounts of iron the next steps are:

1) to determine which ligands are involved in iron binding. MAO is reported to contain 8 cysteine residues per 100,000 mw, and these appear to be essential for activity. MAO would be titrated with sodium mersalyl to determine whether iron was released in this process,

2) to determine the function of iron - MAO preparations have been reported which have good activity but widely varying amounts of iron. Possibly in vitro assays do not measure the role of iron in this enzyme. For example, iron may participate in electron transfer to an intermediate carrier in the mitochondria rather than to $O_2$.

3) to determine whether the activity of purified MAO can be improved by using conditions which might reconstitute an iron or iron-sulfur center.

Keyword Descriptors: monoamine oxidase iron-sulfur protein deamination hypertension

Honors and Awards: None

Publications: None

Project No. Z01 HL 01861-03 HE
1. Hypertension-Endocrine Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: P-Chloromethamphetamine and Tryptophan Hydroxylase

Previous Serial Numbers: NHLI-277, NHLI-278

Principal Investigator: Margaret Walker, Ph.D.

Other Investigators: Walter Lovenberg, Ph.D.
Hans G. Baumgarten, M.D., Ph.D.

Cooperating Units: None

Project Description:

Objectives: Tryptophan hydroxylase is the rate-limiting enzyme in the synthesis of serotonin. Inhibition of tryptophan hydroxylase activity by chlorinated amphetamines has been shown to be one reason for the serotonin depletion caused by these drugs. Because tryptophan hydroxylase inhibition and serotonin depletion are very long lasting despite the short half-life of these drugs in the body, it was thought that the chlorinated amphetamines might have a neurotoxic effect similar to that caused by 5,6- or 5,7-dihydroxytryptamine. Since p-chloromethamphetamine (PCMA) has been used as an antidepressant in the past, and since structurally similar drugs are still in use as appetite suppressants, it seemed necessary to further examine its effect on serotonergic neurons.

Methods: Male Sprague-Dawley rats (150 to 200 gm) were given i.p. injections of either saline or PCMA (10 mg/kg). Rat brains were removed and dissected as described by Victor, Baumgarten and Lovenberg (J. Neurochem. 22: 541, 1974). Brain regions were stored in liquid $N_2$ until assayed, and tryptophan and tyrosine hydroxylase were assayed as described in previous reports.

Major Findings: Although chlorinated amphetamines have been shown to cause an immediate and long-lasting inhibition of tryptophan hydroxylase activity, a comparison of the effects in various brain regions had not been done. In our studies we observed the following:

1) When the time course of tryptophan hydroxylase inhibition was examined in brain regions rich in serotonergic nerve endings (i.e. the septum, striatum and cerebral cortex) there was approximately 30% inhibition after only 3 hours, and maximum inhibition occurred at approximately 24 hours.

2) In brain regions containing cell bodies of serotonergic neurons (the mesencephalic tegmentum and pons medulla) inhibition of tryptophan hydroxylase was not seen until 24 or 48 hours after injection of PCMA.

3) Animals pretreated with chlorimipramine, a serotonergic uptake inhibitor, did not show tryptophan hydroxylase inhibition in any region at either 3 or 6 hours following PCMA treatment (longer time intervals were not examined).

4) Tyrosine hydroxylase activity was not significantly affected.

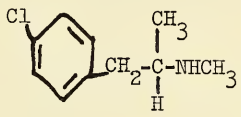
These studies suggest that PCMA is being taken up into serotonergic neurons specifically. It appears to be concentrated at the nerve endings, where it has an immediate effect. The delayed effect on cell body areas may be due to retrograde degeneration of axons and cell bodies.

Significance to Biomedical Research and Institute Program: 1) The use of chlorinated amphetamines as antidepressants in the past and the fact that structurally related drugs are presently being used as appetite suppressants make it essential to determine the mode of action of these drugs and their possible neurotoxicity.

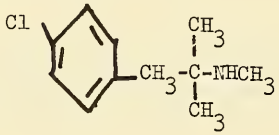
2) Compounds which can selectively inactivate serotonergic neurons would be useful tools in elucidating the role of serotonin in the brain. PCMA does not completely inactivate tryptophan hydroxylase. It shows a different pattern of inhibition than 5,7-dihydroxytryptamine, for instance, and therefore might be used in combination with this or other drugs to further deplete serotonin.

Proposed Course of Project: The appetite suppressant chlorphentermine is structurally similar to PCMA, and it is an effective inhibitor of serotonin uptake.

PCMA




CHLORPHENTERMINE




It has been reported that chlorphentermine does not deplete serotonin in whole rat brain, however, and the effect on tryptophan hydroxylase has not been studied.

We will examine the effect of chlorphentermine on tryptophan hydroxylase activity and serotonin levels in various regions of the rat brain to determine whether there is an effect which is masked in the whole brain.

Keyword Descriptors: p-chloroamphetamine serotonin tryptophan hydroxylase chlorphentermine neurotoxin

Publications: None

Project No. Z01 HL 01900-02 HE
1. Hypertension-Endocrine Branch
2. Section on Experimental Therapeutics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Prostaglandins in Renal and Vascular Physiology

Previous Serial Numbers: NHLI-260(c), 265(c)

Principal Investigator: Robert E. Bowden, M.D.

Other Investigators: Harry R. Keiser, M.D.
John J. Pisano, Ph.D.
John R. Gill, Jr., M.D.
Michael J. Andrews, Jr., M.D.
Robert A. Guyton, M.D.

Cooperating Unit: Surgery Branch, National Heart and Lung Institute

Project Description:

Objectives: 1) Further development of methodology for measuring prostaglandins (PG) in biologic fluids in subnanogram range, and 2) apply these methods to the study of the role of prostaglandins in renal physiology and pathology in hypertension.

Methods: 1. Radioimmunoassay. Assays have been developed as reported previously from this laboratory which can measure reliably $PGA_1$, PGE and PGF with a sensitivity of 150 to 200 pg.

2. Conventional methods of extraction and chromatography of prostaglandins proved to be unsuitable for the radioimmunoassay of many prostaglandins. Consequently, methods utilizing Sephadex LH-20 chromatography for the purification of urinary extracts were developed and proved to be satisfactory for the radioimmunoassay of PGE and PGF.

Major Findings: A. Humans

1. PGE excretion in normal and hypertensive subjects: The range of urinary PGE excretion for normals in our laboratory is from 24 to 45 ng/hr when the subject is eating a diet with 109 mEq sodium daily. PGE excretion increases 20 to 30% on changing from supine to standing position. Patients with essential hypertension (2), renal artery stenosis (2), Bartter's syndrome (5) and idiopathic postural hypotension (1) were studied.

| Patient | Diagnosis | Age (yr) | PGE Excretion (ng/hr) 24 hr | Supine (4 hr) | Standing (4 hr) |
|---|---|---|---|---|---|
| L.G. | Renal artery stenosis | 30 | 20.1 | 10.3 | 7.3 |
| R.A. | Renal artery stenosis | 25 | 26.1 | 29.3 | 19.5 |
| G.A. | Essential hypertension | 32 | 34.2 | --- | --- |
| O.H. | Essential hypertension | 40 | 17.5 | --- | --- |
| B.B. | Bartter's syndrome | 36 | 52.5 | --- | --- |
| D.D. | Bartter's syndrome | 15 | 25.0 | 11.0 | 19.5 |
| M.J. | Bartter's syndrome | Child | 50.2 | 12.5 | 34.0 |
| K.B. | Bartter's syndrome | Child | 40.0 | 25.2 | 43.0 |
| J.E. | Bartter's syndrome | 32 | 105.2 | --- | --- |
| L.H. | Idiopathic postural LBP | 22 | 39.5 | 42.9 | 15.6 |

These data show that patients have a wider range of PGE excretion than normals. Patients with renal artery stenosis tend to excrete lower amounts of PGE, as did one patient with essential hypertension. Patients with Bartter's syndrome tend to excrete higher levels of PGE than normal. Of considerable interest is the finding that 3 patients had a decrease in PGE excretion when changing from the supine to the standing position. Two of these patients had renal artery stenosis, and one had idiopathic postural hypotension, conditions in which one would expect a greater than normal decrease in renal blood flow in changing from the supine to the standing position.

2. Studies using P-113, a competitive antagonist of angiotensin, were performed in the two patients with renal artery stenosis.

| Patient | PGE Excretion (ng/hr) Supine (4 hr) | P-113 (4 hr) |
|---|---|---|
| L.G. | 10.3 | 26.0 |
| R.A. | 29.3 | 21.6 |

One patient had a marked increase in PGE excretion with P-113 while the other had a 26% decrease. The former response is unexpected. The latter response is the expected one since angiotensin II has been reported to increase PGE release. P-113 by blocking the action of angiotensin should decrease PGE excretion. However, the latter patient's response is complicated by the fact that she was also receiving propranolol.

3. One patient, L.G., was studied under identical conditions 5 months apart. PGE excretion (ng/hr) on 8/14/74 was 18.5 (supine), 6.3 (standing) and on 1/1/75 was 10.3 (supine) and 7.3 (standing). Thus the level of PGE excretion appears to remain the same from month to month in the same patient.

4. Two patients, L.H. (postural hypotension) and B.B. (Bartter's syndrome), were treated with Indomethacin, a prostaglandin synthetase inhibitor, at a dose

of 150 mg daily. This decreased the PGE excretion by 50%. It had no effect on the postural hypotension of the first patient but was associated with a significant increase in serum potassium in the second patient.

5. Levels of PGA and PGE were measured in renal venous blood from patients undergoing catheterization of the renal veins for the diagnosis of renal artery stenosis. Two patients have been studied, one with classic renal artery stenosis and a second with an ectopic kidney but with low renin hypertension. Plasma PGE and PGA were obtained from the right and left kidneys and the inferior vena cava in the initial control period and after stimulation with Hydralazine (a potent vasodilator which stimulates the production of renin). Plasma levels of 50 to 70 pg/ml of both PGE and PGA were found and were not different in the 3 sites, before or after Hydralazine. These values are close to the lower limits of detectability of our assay.

B. Dogs

1. Effects of saline infusions and Indomethacin on PGE excretion in hypophysectomized mongrel dogs anesthetized with pentobarbital. As detailed last year the infusion of lactated Ringer's solution produced a 20 to 50% decrease in PGE excretion compared to control values when the animal was receiving 2.5% D/W. When Indomethacin (4 mg/kg I.V.) was administered PGE excretion fell 50 to 75% and there were increases in renal blood flow, urine volume, sodium excretion and free water clearance. These experiments have been extended to include a control infusion of 2.5% dextrose solution throughout the collection periods. There is a decrease in PGE excretion of 25 to 30% from control levels throughout the course of the experiment. These data indicate that the prostaglandin activity decreases not in response to Ringer's solution, but possibly secondary to volume expansion or time after the operative stresses at the start of the experiment.

2. Effects of Indomethacin in conscious dogs with stenosis of the left renal artery. This is a continuation of the studies started and reported last year (NHLI 265c). Dogs were prepared with a 75% stenosis of the left renal artery and bilateral ureterostomies. After the dogs had recovered for several weeks differential renal function studies were performed while the animals were unanesthetized. We now have complete studies in 8 dogs. Urine volume, PAH clearance, inulin clearance, kallikrein excretion and free water clearance were all reduced significantly (mean reduction of 50%) on the left or stenotic side. There was a highly significant correlation between the differences in kallikrein excretion between the normal and the stenotic kidney and the differences in renal blood flow (PAH clearance) in these animals. There was a similarly significant correlation between differences in kallikrein excretion and differences in inulin clearance. There were no such correlations of kallikrein excretion with urine volume or with excretion of sodium or potassium. There was also a significant reduction in excretion of PGE and PGF on the stenotic side. There was a correlation between PGE and PGF levels for both kidneys. When Indomethacin (2 to 10 mg/kg I.V.) was administered there was an average decrease of 80% in PGE and PGF excretion. There was a significant increase in urine volume and free water clearance from both sides, a significant fall in

renal blood flow on only the stenotic side, and no significant changes in inulin clearance or excretion of kallikrein, sodium or potassium.

3. Bioassay. Attempts at bioassay for PGE in samples of human urine have produced levels that are 30 to 40% of our radioimmunoassay results. However, samples are toxic to assay organs. This toxicity appears to be secondary to residues of organic solvents.

Significance to Biomedical Research and Institute Program: The control of the excretion of prostaglandins is poorly understood. The data presented here are very tentative but suggest that PGE is involved either primarily or secondarily in renal physiology. PGE levels fall with standing in certain patients in whom one would expect a strong vasoconstrictive reaction in the kidney. In one patient given an angiotensin blocker, the levels of PGE rose, perhaps correlating with a competitive reduction in this vasoconstrictive response.

Our data with Indomethacin in conscious dogs are most important. They contradict current dogma which says that PG's are mediators of natriuresis and diuresis in the normal kidney. Those studies were done in either isolated perfused kidneys or in kidneys after extensive surgery. Our studies show that inhibition of PG synthesis in unanesthetized animals does not produce profound changes in blood pressure and renal function. In fact the changes we found, while significant, were relatively small inspite of 85% mean reductions in PG levels. Thus PG's are not the mediators of the excretion of salt and water excretion.

Proposed Course of Project: 1) Studies of PG excretion in patients with various types of hypertension before and during treatment. 2) Studies of PG excretion in patients and normals in whom the renin-angiotensin system is either stimulated or inhibited by either physiologic or pharmacologic means. 3) Study of the effects of Indomethacin in patients with Bartter's syndrome. 4) Study of the interrelationships between the PG's, the kallikrein-kinin system and the renin-angiotensin system.

Keyword Descriptors: prostaglandins urine blood radioimmunoassay column chromatography man dogs hypertension Bartter's syndrome indomethacin

Honors and Awards: None

Publications:

Gimbrone, M.A., Jr. and Alexander, R.W.: Angiotensin II stimulation of prostaglandin production in cultured human vascular endothelium. Science, in press.

Project No. Z01 HL 01901-01 HE
1. Hypertension-Endocrine Branch
2. Section on Experimental Therapeutics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Kinins in Urine

Previous Serial Number: None

Principal Investigator: Valdemar Hial, M.D., Ph.D.

Other Investigators: John J. Pisano, Ph.D.
Harry R. Keiser, M.D.

Cooperating Units: None

Project Description:

Objectives: Kinins are potent endogenous peptides which exert their effects in at least three broad areas: the vascular system, inflammatory processes and in smooth muscle. There are three important kinins: bradykinin, Lys-bradykinin and Met-Lys-bradykinin. The enzymes responsible for the production of kinins from kininogens are known as kininogenases (kallikreins). Kallikrein from plasma releases bradykinin and that from glandular tissues releases Lys-bradykinin. The Met-Lys-bradykinin is of obscure origin. Project objectives were to investigate the presence of three kinins in urine giving special emphasis to estimation and biosynthesis of Met-Lys-bradykinin.

Methods: We developed a method to estimate the three kinins in urine using IRC-50 in a batch technique and a SP-Sephadex C-25 column. The kinins were assayed on an isolated guinea pig ileum. Dipeptidyl aminopeptidase I (DAP I) as well as amino acid analysis was used to characterize Met-Lys-bradykinin. Collections of urine for 24 hours were made from normal subjects into plastic containers using 6N HCl to maintain a pH about 2.

Major Findings: The results are summarized in the following table:

| | μg/24 hr (mean ± SEM) |
|---|---|
| **Men (n=10)** | |
| Bradykinin | 3.4 ± 0.7 |
| Lys-Bradykinin | 7.6 ± 1.1 |
| Met-Lys-Bradykinin | 9.5 ± 1.6 |
| Total Kinins | 20.6 ± 2.47 |
| **Women (n=6)** | |
| Bradykinin | 2.0 ± 0.5 |

| | |
|---|---|
| Lys-Bradykinin | 4.8 ± 1.1 |
| Met-Lys-Bradykinin | 1.0 ± 0.4 |
| Total Kinins | 7.8 ± 1.6 |

In men total kinins average 20.6 µg/24 hr and the amounts of Lys-bradykinin and Met-Lys-bradykinin are essentially the same, each constitutes approximately 40% of the total kinins. In women total kinins average only 7.8 µg/24 hr and while the amount of all kinins is reduced, the major reduction is in Met-Lys-bradykinin. These differences between men and women in total kinins and in Met-Lys-bradykinin are both highly significant with $p < 0.001$.

We have some preliminary evidence from in vitro studies which indicate that Met-Lys-bradykinin is released by action of uropepsin on kininogen fragments in human urine when stored at pH 2.0.

Significance to Biomedical Research and Institute Program: This is the first time the individual kinins in human urine have been quantitated. It is also the first time that Met-Lys-bradykinin has been demonstrated in human urine using several different techniques. These data have shown a striking difference between normal men and women. This focuses our attention on the possible role of other enzyme systems in the production of these potent vasodilators. It also suggests possible controlling influences for the production of Met-Lys-bradykinin.

Proposed Course of Project: Studies to prove the actual source of Met-Lys-bradykinin. These will involve investigation of other urinary enzymes as possible sources of kinins and of the relationships between changes in these enzymes (including kallikrein and urokinase) and the levels of the different urinary kinins.

Keyword Descriptors: human urinary kinins bradykinin Lys-bradykinin Met-Lys-bradykinin dipeptidyl-aminopeptidase I column chromatography bioassay

Honors and Awards: None

Publications: None

Project No. Z01 HL 01902-06 HE
1. Hypertension-Endocrine Branch
2. Section on Experimental Therapeutics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Clinical Investigations of Vasoactive Systems

Previous Serial Number: NHLI-264(c)

Principal Investigator: David Horwitz, M.D.

Other Investigators: Walter Lovenberg, Ph.D.
Michael F. Roizen, M.D.
Horst Brobecker, M.D.
Joseph M. Vinci, M.D.
Harry R. Keiser, M.D.

Cooperating Units: Laboratory of Clinical Science, NIMH

Project Description:

A. Studies of Dopamine-β-Hydroxylase (DBH) in Hypertension

Objectives: DBH is the enzyme that catalyzes the final step in the synthesis of norepinephrine the sympathetic neuromediator. DBH is released from sympathetic nerves, can be measured in plasma and has been proposed as an index of sympathetic activity in man. It has been reported that plasma DBH levels are higher in patients with essential hypertension than in subjects with primary etiologies for their hypertension and that such differences are of diagnostic value. Our past observations suggested that plasma levels of DBH activity were an insensitive reflection of sympathetic activity; further, patients with essential hypertension showed low as well as higher levels of DBH activity. Our recent studies were intended to determine whether sympathetic activity differs in essential hypertensives in comparison with subjects with normal blood pressure or with secondary hypertension. At the same time the usefulness of DBH activity was evaluated by comparison with an established and sensitive test, that of plasma norepinephrine levels.

Methods: Blood pressure was determined in supine and erect postures on eight separate visits to establish mean values and variability for each subject. On one visit blood samples were drawn after 30 and 40 minutes of rest and two and ten minutes of standing. Plasma DBH activity was assayed by the Nagatsu method utilizing the enzymatic conversion of tyramine to octopamine and photometric assay after oxidation of octopamine to p-hydroxybenzaldehyde. Plasma norepinephrine was measured by a newly developed method, that of Roizen et al.; the norepinephrine was enzymatically N-methylated and tritium-labeled through use of [$^{3}$H-methyl]-S-adenosylmethionine and phenylethanolamine-N-methyltrans-

ferase; the radioactive end-product was thereafter extracted and assayed by liquid scintillation spectrometry.

Major Findings: Twenty-eight subjects have been studied (20 with intermittent or sustained essential hypertension, 5 secondary hypertensives, 2 normals, 1 with idiopathic orthostatic hypotension). Mean plasma norepinephrine levels for the 20 essential hypertensives were .25 ng/ml while supine and .52 ng/ml after 10 minutes of standing (+108%); the corresponding values for DBH activity were 43 and 46 (+7%) units. The correlation between resting levels of plasma norepinephrine and DBH activity was weak and not statistically significant (Spearman rank correlation coefficient 0.4, probability of chance occurrence > .05). Six subjects underwent the stimulus of a four hour period of standing; at the end of the period plasma DBH levels had risen only 14% (51 units pre, 58 post) whereas plasma norepinephrine levels were 111% higher (0.21 ng/ml pre, .45 post). The findings in a patient with advanced idiopathic orthostatic hypotension were of special interest in that they revealed negligible and unresponsive plasma norepinephrine levels (.05, .07, .06 ng/ml) in the presence of normal DBH activity (48 Nagatsu units).

Dopamine-β-hydroxylase levels have been determined in nine patients with pheochromocytoma; three of the subjects showed values which were above the normal range and four were at the upper limits of normal. Postoperative values were determined for six subjects and revealed a 56% fall from an initial mean level of 82 Nagatsu units. Serial blood samples drawn after removal of the tumor revealed an exponential decline of plasma DBH activity with a half-life of 8 hours.

Significance to Biomedical Research and Institute Program: The recent results support our previous conclusion that plasma DBH activity is a relatively insensitive index of sympathetic nervous system function in man. The half-life of plasma DBH has been measured for the first time in patients with pheochromocytomas. The prolonged half-life of DBH correlates with the muted responsiveness of this index.

Proposed Course of Project: Additional subjects will be studied to permit valid comparisons of essential hypertensives with normal subjects and patients with secondary forms of hypertension.

B. Effect of Dietary Potassium on Urinary Excretion of Kallikrein

Objectives: Urinary kallikrein is an enzyme that forms the vasodilator kinin, kallidin, from a plasma substrate. We have previously shown that urinary kallikrein is increased by salt deprivation and by administration of sodium-retaining steroids. The object of the present study was to determine whether urinary excretion of kallikrein is influenced by dietary intake of potassium and whether this vasodilator system responded similarly in normotensive and hypertensive subjects.

Major Findings: The effects of three levels of potassium intake (85 mEq for 5 days, 185 mEq for 7 days and 25 mEq for 10 days) on kallikrein excretion

were observed in 16 normal subjects and eight patients with uncomplicated essential hypertension; intake of sodium was kept at 110 mEq throughout. Urinary kallikrein was measured by the radiochemical assay of Beaven et al. Urinary excretion of kallikrein was higher with the 185 mEq than with 25 mEq potassium intake for each subject, was usually proportional at intermediate levels of intake, and paralleled aldosterone excretion. Changes in kallikrein excretion were gradual reaching maximum levels after 5 to 7 days of high potassium intake and minimum levels after 6 to 10 days of low potassium intake. Responses were qualitatively similar for normotensive and hypertensive subjects. Mean urinary excretion on the last day of each period is shown in the following table:

| | | 85 mEq | 185 mEq | 25 mEq |
|---|---|---|---|---|
| Kallikrein (units/day) | 16 Normals | 10.8 | 19.1 | 5.8 |
| Kallikrein (units/day) | 8 Hypertensives | 8.8 | 13.9 | 6.1 |
| Aldosterone (µg/day) | n = 19 | 16.1 | 28.8 | 4.2 |
| Potassium (mEq/day) | n = 24 | 82 | 155 | 26 |

Mean values for kallikrein or aldosterone differed significantly ($p < .01$) for each period. In one additional subject the high potassium intake was prolonged to two weeks and the low potassium period to four weeks; the corresponding levels of kallikrein excretion were sustained for the duration of each period.

Significance to Biomedical Research and Institute Program: Potassium is shown to be a major determinant of the urinary excretion of both kallikrein and aldosterone. These findings support the hypothesis that alterations in aldosterone levels mediate changes in kallikrein excretion.

Proposed Course of Project: Direct measurements of urinary and plasma kinin levels will be attempted and relationships to renal function and alterations in salt metabolism and sympathetic nervous system activity will be investigated.

Keyword Descriptors: dopamine-β-hydroxylase norepinephrine sympathetic nerve activity human hypertension urinary kallikrein aldosterone dietary potassium

Honors and Awards: None

Publications:

1. Margolius, H.S., Horwitz, D., Pisano, J.J., and Keiser, H.R.: Urinary kallikrein excretion in hypertensive man. Relationships to sodium intake and sodium-retaining steroids. Circulation Res. 35: 820-825, 1974.

2. Lovenberg, W., Bruckwick, E.A., Alexander, R.W., Horwitz, D., and Keiser, H.R.: Evaluation of serum dopamine-β-hydroxylase activity as an index of sympathetic nervous activity in man. In Usdin, E. (Ed.): Neuropsychopharmacology of Monoamines and Their Regulatory Enzymes. New York, Raven Press, 1974, pp. 121-128.

Project No. Z01 HL 01903-01 HE
1. Hypertension-Endocrine Branch
2. Section on Experimental Therapeutics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Plasma Prekallikrein in Hypertension

Previous Serial Number: None

Principal Investigator: Teruhiro Nakada, M.D.

Other Investigators: Harry R. Keiser, M.D.

Cooperating Units: None

Project Description:

Objectives: A low level of urinary kallikrein excretion in patients with essential hypertension was first reported by Elliot and Nuzum as long ago as 1934. Since then, literature dealing with the relationship between high blood pressure and the kallikrein-kinin system has been scanty. The main objective of this research was to clarify the relationship between plasma prekallikrein and blood pressure with special reference to the influence of dietary sodium intake.

Methods: Seventeen normotensive volunteers and 22 hypertensive patients were studied. Blood samples were drawn from each subject when he was taking either an ad lib diet or one with a controlled level of sodium. In patients with renovascular hypertension, Hydralazine (a potent vasodilator) was administered to examine its effect on the level of plasma prekallikrein.

In animal experiments, plasma prekallikrein was assayed in dogs eating either a regular diet or a diet high in sodium (180 mEq/day) and in dogs with unilateral renal artery stenosis. In Wistar/NIH rats experimental hypertension was produced by administration of desoxycorticosterone (DOC) and tap water with 1% NaCl. Groups of rats on either DOC or 1% NaCl or normal diet were used as controls. Plasma prekallikrein was determined by a modification of the radiochemical assay using p-tosyl-L-arginine methylester (TAMe) as originally developed by Beaven and Pisano. One unit of kallikrein activity is that amount of TAMe esterase activity found in 1.0 ml of a standard acetone-activated human plasma incubated for 30 minutes at room temperature.

Major Findings: There was a small but significantly ($p < .05$) lower level of plasma prekallikrein in patients with essential hypertension ($2.22 \pm 0.08$ units/ml, mean $\pm$ S.E., n=9) when compared with normotensive subjects ($2.56 \pm 0.13$ units/ml, n=17). There was no significant difference in the level of plasma prekallikrein between normotensive subjects and patients with renovascular hypertension ($2.52 \pm 0.07$ units/ml, n=11). Plasma prekallikrein values

were the same in normotensive men (2.61 ± 0.12 units/ml, n=10) and women (2.48 ± 0.26 units/ml, n=7). The effect of dietary sodium intake on plasma prekallikrein was as follows (n=2): low sodium intake (9 mEq/day), 3.48 ± 0.33 units/ml; normal sodium intake (109 mEq/day), 2.42 ± 0.15 units/ml; high sodium intake (259 mEq/day), 2.63 units/ml. Intravenous injection of 20 mg of Hydralazine reduced the plasma prekallikrein level significantly ($p < 0.005$) (pre-infusion value 2.42 ± 0.07 units/ml, n=6; post-infusion value 1.85 ± 0.09 units/ml, n=6).

In dog studies there was no significant difference of plasma prekallikrein among the three groups: normal controls 0.45 ± 0.10 units/ml, mean ± S.E., n=8; high sodium diet 0.56 ± 0.04 units/ml, n=4; and renovascular hypertension 0.47 ± 0.04 units/ml, n=6. In rat experiments repeated administration of DOC (12.5 mg/100 g body weight) elevated plasma prekallikrein from 2.99 ± 0.28 units/ml (n=8) to 3.84 ± 0.13 units/ml, (n=13) on the tenth experimental day and this value increased further to 4.29 ± 0.16 units/ml (mean ± S.E., n=12) on the 30th experimental day ($p < 0.005$). However, administration of 1% sodium chloride with DOC did not increase plasma prekallikrein levels on the tenth experimental day (3.09 ± 0.13 units/ml, n=10) and the slight increase on the 30th experimental day (3.42 ± 0.15 units/ml, n=7) was not statistically significant. A high sodium diet did not significantly change levels of plasma prekallikrein in rats. On the other hand, a low sodium diet increased plasma prekallikrein significantly from control values of 2.99 ± 0.28 units/ml, n=8, to 3.82 ± 0.14 units/ml, mean ± S.E., n=9, on the tenth experimental day ($p < 0.025$), however, the values returned to control level on the 30th experimental day (2.89 ± 0.11 units/ml, mean ± S.E., n=8)

Significance to Biomedical Research and Institute Program: The finding of a lower level of plasma prekallikrein in hypertensive patients is important since it suggests that the plasma kallikrein-kinin system is more active in hypertension. Review of this data indicates that many of these hypertensives were on therapy and had normal blood pressures when the blood sample was drawn. Thus, this finding will need further verification. It is clear that a low sodium diet produces a significant increase in plasma prekallikrein. This raises an important point. We have shown previously that urinary kallikrein is increased by a low sodium diet. The higher plasma prekallikrein could reflect: 1) decreased activity of the plasma kallikrein-kinin system, 2) presence of some renal kallikrein in plasma, or 3) an increase in formation of plasma prekallikrein due to the action of aldosterone (as we have shown for urinary kallikrein). The fall in plasma prekallikrein after Hydralazine suggests that the plasma kallikrein-kinin system may be a mediator in drug induced vasodilation. These factors all have important bearing on the role of the kallikrein-kinin system in normal physiology and in hypertension.

Proposed Course of Project: Plasma prekallikrein will be assayed in more untreated patients with essential hypertension. Plasma kininogen, another indicator of the activity of the plasma kallikrein-kinin system, will be assayed in the same patients to help explain our findings in human hypertension. The effects of various vasodilator drugs on plasma prekallikrein will be studied in man and rat.

Keyword Descriptors: plasma prekallikrein man dog rat hypertension dietary effects dexoxycorticosterone renal artery stenosis

Honors and Awards: None

Publications: None

Project No. Z01 HL 01904-01 HE
1. Hypertension-Endocrine Branch
2. Section on Experimental Therapeutics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Renal Kallikrein and Plasma Kininogen in Hypertension

Previous Serial Number: None

Principal Investigator: Frank Perez Acuna, M.D.

Other Investigators: Harry R. Keiser, M.D.

Cooperating Units: None

Project Description:

Objectives: 1. Develop a method for the assay of kallikrein in renal tissue. 2. Determine if there is a correlation between amounts of kallikrein in renal tissue and in urine in several different animal models of hypertension. 3. Determine if values of plasma kininogen are abnormal in hypertensive humans and animals.

Methods: After extensive experimentation the following technique was developed for measuring kallikrein in renal tissues. Rats were anesthetized, the aorta was ligated above the renal arteries and the kidneys were perfused in situ for 10 minutes with cold isotonic saline and 3.1% sodium citrate to flush out all plasma and its kallikrein. The kidneys were removed and the cortex was isolated, weighed, and homogenized in 0.25 M sucrose, pH 7.4. After centrifugation at 40,000 rpm for 30 minutes the supernatant was filtered through Sephadex G-25. The filtrate was assayed for kallikrein activity by incubation with semipurified dog plasma kininogen and detection of kinin released by bioassay on guinea pig ileum. The incubations were performed either directly in the bioassay bath or in separate tubes, the contents of which were boiled and later added to the bioassay bath.

Urinary kallikrein was measured via the radiochemical esterolytic assay using $^{3}$H-tosylarginylmethyl ester ($^{3}$H-TAME) as described in previous reports.

Plasma kininogen was assayed by incubation of aliquots of human or rat plasma with appropriate excesses of either partially purified human or rat urinary kallikrein. The kinins generated were assayed on the guinea pig ileum.

Major Findings: A surprisingly large amount of kininase activity was associated with the kallikrein from rat kidneys. This made assays of kallikrein invalid because of rapid destruction of kinins. This kininase activity was only partially removed by gel filtration. Various inhibitors of kininase were tried and 8-hydroxyquinoline ($3 \times 10^{-3}$M) was able to inhibit about 70% of the

kininase activity. Using the inhibitor we were unable to find any differences in kallikrein activity in kidneys of spontaneously hypertensive rats and the normotensive Kyoto/Wistar control rats. When the esterolytic assay for kallikrein was applied to the renal cortical homogenates it was apparent that esterolytic activity was about 5 times greater than biologic activity. Thus the nonspecific esterolytic method could not be used. Similar studies using kidneys from rats made hypertensive by a clip on one renal artery or their sham-operated controls showed no differences in levels of kidney kallikrein. Again the problems with kininases were only partially overcome and the overall recovery of kallikrein activity was poor.

When the activity of the kallikrein-kinin system changes there should be appropriate changes in the substrate, i.e. plasma kininogen. Thus the level of plasma kininogen should provide an inverse indicator of kallikrein system activity. Plasma kininogen levels averaged 2.41 $\pm$ 0.45 micrograms kallidin/ml plasma (mean $\pm$ S.E.M.) in normal humans (n=4). In two subjects kininogen did not change significantly after the subject stood for 4 hours. There were no apparent differences in plasma kininogen levels when subjects were tested for 5 to 7 days each on diets of 109, 9, or 259 mEq of sodium daily.

Hypertensive patients (n=5) on therapy had levels of plasma kininogen (1.69 $\pm$ 0.12) that averaged 29% less than normal controls. When 2 patients with essential hypertension were given acute infusions of Hydralazine (20 mg I.V.) they had 15 and 50% increases in plasma kininogen within 30 minutes, during the acute antihypertensive effect of the drug.

Significance to Biomedical Research and Institute Program: The measurement of renal kallikrein is vital to our understanding of this vasodilator system in salt and water homeostasis and in hypertension. These studies of renal kallikrein, while negative, have indicated that other approaches must be used to solve this problem.

The studies of plasma kininogen are very preliminary. However, they indicate that plasma kininogen may provide a useful index of the activity of the kallikrein-kinin system in man and animals. The finding of lower levels of kininogen in treated hypertensives must be extended to untreated hypertensives. But it suggests that this vasodilator system may be attempting to compensate for the hypertension in these patients. The increase in kininogen to infusion of a vasodilator suggests that this system may be responding or buffering acute changes in the sympathetic nervous system or in the renin-angiotensin system. This provides further clues to interactions between these potent vasoregulatory systems.

Proposed Course of Project: The principal investigator is returning to Venezuela. The work on renal kallikrein will stop. The work on plasma kininogen will be continued by others in our group using newer technology.

Keyword Descriptors: rat renal kallikrein bioassay kininase activity human plasma kininogen dietary effects hydralazine

Publications: None

Project No. Z01 HL 01905-05 HE
1. Hypertension-Endocrine Branch
2. Section on Experimental Therapeutics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Urinary Kallikrein and Kinin

Previous Serial Number: NHLI-266(c)

Principal Investigator: Joseph M. Vinci, M.D.

Other Investigators: David Horwitz, M.D.
Harry R. Keiser, M.D.
John J. Pisano, Ph.D.
Robert Brown, M.D.*
Robert Rhamey, M.D.*
John Foster, M.D.*

Cooperating Units: *Department of Medicine
Vanderbilt University

Project Description:

Objectives: To study the role of the kallikrein-kinin system in normal and hypertensive humans and animals.

Methods: 1. Urinary kallikrein excretion is measured with a previously described radiochemical assay [NHLI-79(c)].

2. Urinary kinin is measured by a radioimmunoassay developed by this laboratory using an antibody which binds bradykinin, kallidin and methionyl-lysyl-bradykinin, thus giving a measure of total immunoreactive kinins.

3. Urinary kallikrein was measured in normal and hypertensive man on 109 mEq, 9 mEq and 259 mEq daily sodium intake.

4. On a 109 mEq Na diet, fludrocortisone, 0.5 mg/day, was given and kallikrein excretion measured. Spironolactone, 400 mg/day, was given to normals on a 9 mEq sodium diet and to patients with primary aldosteronism.

5. Urinary kallikrein and kinin were measured in normal subjects and in patients with essential hypertension during a potassium intake of 85 mEq, 185 mEq and 25 mEq daily with a constant sodium intake.

6. Urinary kallikrein was determined in samples from 31 patients undergoing differential renal function studies as part of an evaluation for renovascular hypertension at the Hypertension SCORE Program at Vanderbilt University. The patients also had complete evaluation including rapid sequence intravenous

pyelography, radioisotopic renography, renal arteriography and determination of plasma renin activity in renal veins.

Dogs: 1. Measurements of urinary kallikrein and kinin were performed from each ureter in unanesthetized dogs with bilateral ureterostomies and stenosis of the left renal artery and related to simultaneous measurements of PAH clearance, inulin clearance, urine flow, osmolality, urinary sodium and potassium excretion.

2. The effects of intravenous administration of minoxidil and the angiotensin converting enzyme (SQ 20,881) on the urinary kallikrein-kinin system were determined.

Major Findings: Humans

1. Relationships to Sodium Intake and Sodium-Retaining Steroids

a) Normal man: When sodium intake was changed from 109 mEq/day to 9 mEq/day, daily kallikrein increased progressively in each of 13 subjects to a mean maximal value that was 271% of control by day 7. An increase in sodium intake to 259 mEq/day resulted in the return of kallikrein excretion to control values. Administration of fludrocortisone to 4 subjects on a 109 mEq Na diet for 10 days resulted in increased kallikrein excretion to a mean maximal value that was 203% of control. In two subjects on a diet containing 9 mEq Na/day, elevated kallikrein excretion decreased on 400 mg spironolactone daily. These findings demonstrate that the kallikrein-kinin system responds to sodium-retaining steroids whether of endogenous or exogenous origin.

b) Hypertensives: Eleven patients with essential hypertension excreted significantly less ($p < 0.001$) kallikrein than did 13 normal subjects on a 109 mEq diet. On a 9 mEq Na diet kallikrein excretion increased in the majority of patients with essential hypertension but remained significantly less ($p < 0.001$) than in normal subjects. Three patients with primary aldosteronism exhibited a kallikrein excretion that was seven-fold higher ($p < 0.001$) than patients with essential hypertension and their kallikrein excretion was unchanged when dietary sodium was changed. Their kallikrein excretion, however, was decreased by treatment with spironolactone.

2. Relationship to Potassium Intake

Thirteen normals receiving a 185 mEq K and 109 mEq Na diet showed a 216% mean maximal increase in urinary kallikrein excretion compared to control values obtained on an 85 mEq K and 109 mEq Na diet. With reduction of K intake to 25 mEq, there was a 66% mean maximal decrease in kallikrein excretion from control values. Seven essential hypertensives showed a maximum increase in kallikrein excretion of 198% on high potassium intake and an 80% mean maximum reduction in urinary kallikrein excretion on low potassium excretion. Again urinary kallikrein appears responsive to the effective level of circulating mineralocorticoid activity, this time when the latter is altered by changes in potassium intake a known potent stimulus to aldosterone production.

In preliminary observations when urines from the control period of this study were assayed for kinins there was a highly significant ($p < 0.01$) correlation ($r = 0.785$) between kinin excretion and kallikrein excretion ($n=18$).

3. Renovascular Hypertension

Very little or no kallikrein was detected in the urine from 10 of the 31 patients. Five of these patients had unilateral renovascular disease and 5 had disease which was considered to be bilateral (in 3 it was of equal severity from side to side and in 2 it was unequal). In addition, there were 4 patients with low or undetectable kallikrein in the urine from only one kidney. In all 4 of these patients the renovascular disease was unilateral and the involved kidney was the one with low or undetectable kallikrein. This finding of low or undetectable levels of kallikrein is very rare in our experience. Of over 1000 adults tested only 3 had very low levels of urinary kallikrein and all 3 were hypertensives. Only 2 of 603 normal children had very low urinary kallikrein. These studies are preliminary but they show that in a significant number of patients there is little or no kallikrein detectable in the urine from one or both kidneys. In addition, they show that the kidney affected with renovascular disease generally excretes reduced or undetectable amounts of kallikrein.

Dogs

Unanesthetized dogs with bilateral ureterostomies and stenosis of the left renal artery of greater than 75% showed:

1) 2.31 times the urine flow on the right compared to the left, 2) 2.11 times the PAH clearance on the right compared to the left, 3) correlation of changes in renal blood flow and changes in kallikrein with a correlation coefficient of 0.94 ($p < 0.01$), 4) no change in kallikrein excretion in response to Indomethacin.

Significance to Biomedical Research and Institute Program: Collectively the above data support the hypothesis that the renal kallikrein-kinin system is involved in hypertensive disease and in salt and water homeostasis.

The above data show that urinary kallikrein is correlated with renal blood flow and that both are reduced in renovascular hypertension in man and dogs. Drugs which increase renal blood flow appear to increase urinary kinin excretion acutely without affecting kallikrein excretion.

Proposed Course of Project: 1. Continue to test the hypothesis that the kallikrein-kinin system is dependent on mineralocorticoid activity and consequently on the renin-angiotensin system. This will involve extension of our measurements to the plasma kallikrein-kinin system and the study of this and the urinary kallikrein-kinin system in induced hyper-reninemic states.

2. Further characterization of the kallikrein-kinin system in low, normal and high renin patients.

3. Study the effects of hydralazine, a peripheral vasodilator, on the kallikrein-kinin system in dog and man.

4. Study the effects of the angiotensin converting enzyme inhibitor, SQ 20,881, on the kallikrein-kinin system in normal renin hypertensives.

5. Study the diagnostic potential of kallikrein excretion in diseases affecting renal cortical blood flow.

6. Determine more precisely the relationship between the kallikrein-kinin system and the prostaglandins.

Keyword Descriptors: human hypertension urinary kallikrein renal artery stenosis potassium intake aldosterone urinary kinins sodium intake

Honors and Awards: None

Publications:

1. Margolius, H.S., Horwitz, D., Geller, R.G., Alexander, R.W., Gill, J.R., Jr., Pisano, J.J., and Keiser, H.R.: Urinary kallikrein excretion in normal man. Relationships to sodium intake and sodium-retaining steroids. Circulation Res. 35: 812-819, 1974.

2. Keiser, H.R., Margolius, H.S., Brown, R., Rhamey, R., and Foster, J.: Urinary kallikrein in patients with renovascular hypertension. In Pisano, J.J. and Austen, K.F. (Eds.): Chemistry and Biology of Kallikrein-Kinin System in Health and Disease. Washington, D.C., United States Government Printing Office.

Project No. Z01 HL 01906-02 HE
1. Hypertension-Endocrine Branch
2. Section on Experimental Therapeutics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Urokinase Excretion and Function

Previous Serial Number: NHLI-267(c)

Principal Investigator: Gilbert M. Wilcox, M.D.

Other Investigators: Harry R. Keiser, M.D.
John J. Pisano, Ph.D.
Valdemar Hial, M.D., Ph.D.
David Horwitz, M.D.

Cooperating Units: None

Project Description:

Objectives: Urokinase (UK) is a urinary proteolytic enzyme which activates plasminogen to plasmin and thereby dissolves fibrin clots. Methods to measure UK have utilized either: measurement of the area of fibrin clot dissolved when a drop of UK containing solution is applied to a fibrin-coated slide which contains an excess of plasminogen or measurement of UK esterolytic activity using an appropriate ester substrate. A new radiochemical esterolytic method has been developed using $H^3$-acetyl-glycyl-lysine methyl ester. The first objective was to determine the validity of this simple and highly sensitive assay by comparison with the fibrin plate assay. Subsequent project objectives were to: 1) investigate UK stability in solutions of varying protein and salt content, 2) determine urokinase excretion in normal subjects, 3) determine UK excretion under the following experimental conditions: nephrectomy, experimental hypertension, renal vein thrombosis and in human kidney transplant rejection, and 4) study UK excretion in normal subjects during various maneuvers to determine its relationships with sympathetic nervous system activity and the kallikrein-kinin system.

Methods: Urokinase was assayed using $H^3$-acetyl-glycyl-lysine methyl ester as enzyme substrate; UK action on this synthetic ester splits off $H^3$-methanol which was measured in a scintillation counter. Timed urine collections were made under various experimental conditions and bovine serum albumin was added to each sample to enhance stability. Whole urine was assayed, the radioactivity generated being proportional to the enzyme activity.

Major Findings: 1) Assay of duplicate aliquots of human and rat urine demonstrated that data obtained with the radiochemical assay was analogous with urokinase measured with the fibrin plate assay.

2) The radiochemical assay was highly reproducible with a interassay variability of less than 10% and measured small amounts of UK.(lower limit of sensitivity of 0.2 CTAU/ml).

3) Urokinase was unstable in solutions of low protein or salt concentration; stability was greatly enhanced by raising the protein concentration (addition of albumin or lysozyme) and was essentially independent of salt concentration if albumin concentration was greater than 1 mg/ml.

4) Urokinase excretion in normal volunteers on 109 mEq $Na^+$/100 mEq $K^+$ diet and ad lib activity was: men 8131 $\pm$ 4454 CTAU/24 hr, n=6, (mean $\pm$ SEM); women 7686 $\pm$ 3464 CTAU/24 hr, n=5. Recovery of UK from human urine was 78% $\pm$ 3.5%.

5) Urokinase excretion was measured in control (sham operation) and in rats made hypertensive by unilateral renal artery stenosis: control 530 $\pm$ 96 CTAU/24 hr, n=5, RAS hypertensive 1034 $\pm$ 404 CTAU/24 hr, n=6.

6) The effect of posture on urokinase excretion was studied. Eleven subjects were fed a 109 mEq $Na^+$ 100 mEq $K^+$ diet. Four hour urine specimens were collected on consecutive days always between 5 am and 9 am; only posture was varied. Urokinase excretion was 728 $\pm$ 197 (supine) and 1270 $\pm$ 209 CTA units/4 hr (standing) (mean $\pm$ SEM). This increase was observed in 10 of 11 subjects and was highly significant ($p < .005$). These data show a possible correlation between urinary plasminogen activator excretion and sympathetic nervous system activity.

Significance to Biomedical Research and Institute Program: The radiochemical assay has been validated as a simple and accurate assay for urokinase. The conditions for the stability of urokinase in urine have been established. Our values for urokinase excretion in normals are similar to those reported by others using the fibrin plate assay. Urokinase excretion was increased in rats with renal artery stenosis. This may represent a compensatory response since urokinase may be an additional source of urinary kinins (potent vasodilators). The study of the effects of posture on urokinase excretion suggest a direct relationship with sympathetic nervous system activity. Science continues to search for a good integrated indicator of sympathetic nervous system activity. Urokinase excretion may provide such an indicator.

Proposed Course of Project: Studies will be performed to pursue the relationship between urokinase excretion and sympathetic nervous system activity. These will include infusion of specific vasoactive amines into normal volunteers and study of the effects of sympatholytic drugs in hypertensives. The role of urokinase in producing kinins will be pursued by measuring both substances under these various experimental procedures.

Keyword Descriptors: urokinase plasminogen activator human and rat urine postural effects renal artery stenosis sympathetic nervous system fibrin plate assay

Publications: None

Project No. Z01 HL 01941-04 HE
1. Hypertension-Endocrine Branch
2. Physiological Chemistry
3. Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies on the Structure of Villikinin

Previous Serial Number: NHLI-217

Principal Investigator: John J. Pisano, Ph.D.

Other Investigators: Eszter Kokas, Ph.D.

Cooperating Units: Physiology Department
University of North Carolina
Medical School

Project Description:

Objectives: To isolate and determine the structure of villikinin, a substance obtained from intestinal mucosa which has a specific stimulant action on intestinal villous motility.

Methods Employed: Canine and porcine intestinal mucosa samples were extracted with trichloroacetic acid (TCA) and the extracts applied to Dowex 50, Bio-Gel P-4 and Bio-Gel P-10 columns. Active fractions were bioassayed in dogs *in vivo*, by the usual method of noting the increase in intestinal villous motility.

Major Findings: At least four peaks with kallikrein-like activity were observed when crude canine villikinin was gel filtered on Bio-Gel P-4 columns. The high molecular weight substance(s) and the two unstable low molecular weight substances were not examined further since their chemical and biological properties proved to be different from villikinin. The main fraction having an approximate MW 2000-3000 was inactivated by leucinaminopeptidase and Nargase. Experiments with carboxypeptidase A were inconclusive because the enzyme unexpectedly affected the bioassay. Upon rechromatography of the active material on Bio-Gel P-10, a single peak of activity was obtained with an apparent MW 2000. This material was inactivated by Pronase. A dose-response curve has been determined for villikinin by both topical and intravenous administration. Porcine mucosa contains material biologically and chemically similar to canine villikinin.

Significance to Biomedical Research and the Program of the Institute: Villikinin appears to be a specific hormone which controls intestinal villous



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motility. Contraction of villi promotes lymph flow and absorption of nutrients from the GI tract.

Proposed Course: To determine if porcine mucosa is a richer source of villikinin. To isolate and determine the structure of villikinin.

Keyword Descriptors: villikinin intestinal villi villous motility polypeptide intestinal mucosa

Honors and Awards: None

Publications: None

Project No. Z01 HL 01942-05 HE
1. Hypertension-Endocrine Branch
2. Physiological Chemistry
3. Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Clinical Biochemistry of the Kallikrein-Kinin System

Previous Serial Number: NHLI-280

Principal Investigator: John J. Pisano, Ph.D.

Other Investigators: Jorge A. Guimaraes, Ph.D.
Kerin Yates, B.S.

Cooperating Units: None

Project Description:

Objectives: To develop procedures for the assay of components of the kallikrein-kinin system. To establish the role of the kallikrein-kinin system in health and disease. To determine if plasmin generates peptides in plasma which potentiate kinin actions.

Methods Employed: Bioassay and radioimmunoassay of kininogen and kinins. Enzymatic digestions of plasma with trypsin, kallikrein and plasmin.

Major Findings: 1) Kininogen Assay: The classical method for the assay of kininogen involves the bioassay of bradykinin generated by the action of an excess of trypsin added to heated plasma. It has been suspected by some but not generally recognized that trypsin also generates peptides which potentiate the action of bradykinin when bioassayed with the rat uterus of guinea pig ileum. To determine if trypsin does, in fact, produce potentiating peptides we first liberated all the kinin from kininogen in a plasma sample by prolonged incubation with an excess of human urinary kallikrein. Liberated kinin was inactivated in the process by kininases in the plasma. Trypsin or plasmin was then added to the sample to produce the suspected potentiating peptides and the recovery of added bradykinin determined. A potentiation factor was calculated as $PF = \frac{\text{recovered bradykinin}}{\text{added bradykinin}}$. Both trypsin and plasmin produced peptides which potentiated the action of bradykinin on the guinea pig ileum; PF = 2.85. Taking this factor into consideration 3.00 µg of bradykinin is released by the kininogen in 1 ml plasma. Other laboratories not aware of the extent of potentiation have erroneously reported 8-10 µg bradykinin. The finding that physiological levels of plasmin generates potentiating peptides has considerable significance since it is likely that kinin generation and



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plasminogen activation simultaneously occurs in a number of pathophysiological conditions.

Human plasma contains a kininogen type preferred by plasma kallikrein. It accounts for 15-38% of the total kininogen. Glandular kallikrein is about equally active on all the kininogen types. See report # Z01 HL 1944-18 HE.

Kinin released from kininogens was also determined by radioimmunoassay. The good agreement with the bioassay indicates that the more practical radioimmunoassay technique now can be used to assay kininogens.

Plasma kallikrein has been determined in 12 normal subjects. The mean plasma level of four females taking oral contraceptives was 0.65 TU/ml, two females not on the pill, 1.22 and 6 males, 1.14. The lower level of plasma kallikrein in females on the pill is of considerable interest because of the high incidence of hypertension in this group.

Significance to Biomedical Research and the Program of the Institute: Assay of total kininogens and the plasma-kallikrein-preferral kininogen type will be essential to the evaluation of the pathophysiologic role of the kallikrein-kinin system. The generation of bradykinin potentiating peptides by plasmin points to an important interrelationship of the fibrinolytic and kallikrein-kinin systems.

Proposed Course: To use the kininogen assay in studies on the pathophysiologic role of the kallikrein-kinin system especially in hypertensive diseases. To study the interrelationship of the kallikrein-kinin fibrinolytic and sympathetic nervous systems. To study the plasma kallikreinin females taking oral contraceptives.

Keyword Descriptors: kininogen kinin plasmin kallikrein radioimmunoassay

Honors and Awards: None

Publications: None

Project No. Z01 HL 01943-05 HE
1. Hypertension-Endocrine Branch
2. Physiological Chemistry
3. Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Peptide Biochemistry

Previous Serial Number: NHLI-281

Principal Investigator: John J. Pisano, Ph.D.

Other Investigators: Carl L. Zimmerman, M.S.
Hiroshi Nakamura, Ph.D.

Cooperating Units: None

Project Description:

Objectives: To develop necessary methodology for the micro characterization and assay of peptides and amino acids.

Methods Employed: High pressure liquid chromatography, spectrophotofluorometry.

Major Findings: Previous methods for the HPLC analysis of PTH-amino acids formed in the Edman sequence analysis of peptides has been improved by the use of new microparticle columns. Separation of all PTH amino acids with the exception of PTH-met from PTH-val has been achieved by gradient elution from a 25 cm DuPont Zorbax-ODS column. This system is currently in use in the analysis of PTH samples from both manual and automated degradation.

In addition to gradient elution, a method has been developed for the identification of PTH-amino acids by the less expensive and potentially more rapid isocratic elution from two columns: Group A, the most polar PTH derivatives of asp,glu,asn,gln,thr,ser,gly,ala and tyr are resolved on a 25 cm 12 mm column of Zorbax-ODS. The non-polar Group B derivatives: met,val,phe,ile,leu, trp and lys are separated on a DuPont ETH (100 cm x 12 mm). Dinitrophenyl (DNP) amino acids formed by reaction with 1-fluoro-2,4-dinitrobenzene have been analyzed by HPLC. This procedure is the most sensitive and specific method for the analysis of DNP-amino acids and will have its greatest application in the determination of the N-terminal amino acid of polypeptides.

Tryptophan, peptides containing N-terminal tryptophan, tryptamine and certain related compounds react with Fluram to form derivatives with uniquely high fluorescence in strong acid.

A membrane filter assay has been developed for the determination of proteins in the submicrogram range. Proteins are: (1) treated with Fluram in sodium borate buffer (pH 9.0) containing 0.03 M $MgCl_2$, (2) collected on membrane filters, and (3) desorbed from the filters by an acetone-sodium borate buffer (pH 9.0). By measuring the fluorescence intensity of the extract, as little as 0.3 μg of protein can be determined in the presence of reactive low molecular weight substances which do not interfere because they are not retained by the filter.

Significance to Biomedical Research and the Program of the Institute: The development of improved methods for the determination of amino acid sequences of polypeptides, will increase the feasibility of determining the structures of countless naturally occurring biologically active peptides. Better methods for the detection and assay of vasoactive peptides will facilitate the study of their biological roles.

Proposed Course: To develop, perfect and apply essential new methods of the analysis for polypeptides.

Keyword Descriptors: amino acid sequence PTH amino acids DNP amino acids N-Group analysis amino acid analysis fluorometric assay protein assay

Honors and Awards: None

Publications:

Tamura, A., Nakajima, T., Nakayama, T., et al.: Identification of peptides with 1-dimethylaminoaphthalene-5 sulfonyl chloride. Anal. Biochem. 52:595-606, 1973.

Zimmerman, C.L., Pisano, J.J., and Appella, E.: Analysis of amino acid phenylthiohydantoins by high speed liquid chromatography. Biochem. Biophys. Res. Comm. 55:1220-1224, 1974.

Project No. Z01 HL 01944-18 HE
1. Hypertension-Endocrine Branch
2. Physiological Chemistry
3. Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Biochemistry of the Kallikrein-Kininogen-Kinin System

Previous Serial Number: NHLI-284

Principal Investigator: Jack V. Pierce, Ph.D.

Other Investigators: Jorge A. Guimaraes, Ph.D.
Marion E. Webster, Ph.D.
John J. Pisano, Ph.D.
Allen P. Kaplan, M.D.

Cooperating Units: Allergic Diseases Section
Laboratory of Clinical Investigation
National Institute of Allergy and Infectious Diseases

Project Description:

Objectives: Purification of glandular kallikrein and components of the plasma kinin system for characterization purposes and for production of specific antiserums. Preparation of purified specific antibodies for biochemical and chemical studies. Preparation of affinity adsorbents from purified antibodies and antigen for purification and other purposes, such as devising specific biochemical and radioimmunochemical assays. Applications of these purified proteins, affinity adsorbents, and assay methods to studies of human disease states, such as hypertension.

Methods Employed: Protein purification; enzyme kinetics ; bioassay.

Major Findings: 1) Human Plasma Kininogens. a) Kinetics: Two of the highly purified kininogens, B2α and B4β, described in the previous report (Serial No. NHLI-284), were used for kinetic analyses of human urinary and plasma kallikreins, bovine trypsin, and human plasmin. The results are summarized below:

| Enzyme | Kininogen | | | |
|---|---|---|---|---|
| | B2α* | | B4β** | |
| | $K_m$ (μM) | $k_{cat}$ ($sec^{-1}$) | $K_m$ (μM) | $k_{cat}$ ($sec^{-1}$) |
| Human urinary kallikrein | 9.0 | 4.5 | 5.8 | 4.2 |
| Human plasma kallikrein | 115 | 2.7 | 3.7 | 3.2 |
| Bovine trypsin | 23.1 | 0.67 | 27.0 | 0.52 |
| Human plasmin | 23.1 | 0.015 | --- | --- |

* 1.0 mole kinin/72,400 g

** 1.0 mole kinin/106,000 g

These two kininogen forms were chosen because B2α appears to be the major LMW kininogen in human plasma (35% of total) and the form probably isolated by other workers; and because B4β comprises the major part of the HMW kininogens. As shown, human plasma kallikrein sharply discriminates between them. It has about thirty times the affinity for B4β as for B2α; the latter is a very poor substrate for this kallikrein compared with other kallikreins and even with the non-kallikrein, trypsin. B4α and B4γ were like B4β in being very good substrates for plasma kallikrein.

b) HMW Kininogen (B4), a New Clotting Factor: The above results assume a much greater interest in view of the recent discovery that certain patients with "a prolonged activated partial thromboplastin time and an inability to form plasmin. bradykinin or PF/dil" (K.D. Wuepper et al., Flaujeac Trait: Deficiency of Kininogen in Man. Fed. Proc. 34:859 Abs., 1975) lack both LMW and HMW kininogens, but only the latter form can correct the clotting and other defects: "The kinin-forming, intrinsic coagulation and fibrinolytic pathways of plasma and the formation of the permeability globulin PF/dil are dependent upon plasma HMW-kininogen" (op cit.). Two other groups have found patients with a similar defect which is also corrected by fractions containing HMW kininogen. Part of the initial evidence for this hypothesis was obtained by means of monospecific antiserums to LMW human kininogens prepared in our laboratory. We had earlier found immunological identity between LMW and HMW kininogens with these antiserums.

Sheep antiserums to both B4β and B4γ contain at least two classes of antibodies, one directed against the kininogens and the other directed against a non-kininogen group called X, in B4α, β, and γ. We have isolated this second class of antibodies, $Ab_X$, by affinity chromatography of the antiserums on columns of insolubilized LMW kininogen, followed by affinity chromatography of the filtrate on another column of insolubilized B4β + γ. There appears to be a third class of antibodies still to be investigated.

Our various crude and highly purified kininogen fractions have been tested in three ways: 1) by an assay developed by M.E. Webster for an unknown factor required for the activation of unactivated Hageman factor ($HF_i$); 2) by the classical clotting assay (performed by A.P. Kaplan, using Williams plasma

which lacks the Flaujeac trait); and 3) by Ouchterlony double diffusion, using $Ab_X$. The results are summarized below:

| Kininogen | Activity | | |
|---|---|---|---|
| | $HF_i$ Activator | Clotting Time | Ouchterlony |
| B1α, B2α, B3.1α and β | - | - | - |
| B3.2α and β | | | |
| B4α, β, and γ | + | + | + |
| Peak A1 | - | - | - |
| Peak A2 | - | - | - |
| Peak A3 | + | + | + |
| Peak A4 | + | + | + |
| B4γ incubated with: | | | |
| Human plasma kallikrein | + | + | + |
| Human urinary kallikrein | + | N.T.* | + |
| Hog pancreatic kallikrein | + | N.T. | + |
| Bovine trypsin | - | N.T. | +** |
| Human plasmin | - | N.T. | +** |
| Porcine pepsin | - | N.T. | - |

* N.T. = not tested.

** Spurs indicating the presence of kininogen antigen + Kgn-X, instead of only Kgn-X as in B4γ or B4γ treated with kallikreins.

c) Methionyl-lysyl-bradykinin (MLBK), the Peptide Kinin from Human Kininogens: The reaction of several purified human kininogens at pH 2-6 with crystalline porcine pepsin very rapidly gave a kinin peptide which was stable for extended periods to further digestion by pepsin. The peptic peptide had about one-eighth the guinea pig ileum activity of the same amount of kininogen treated with trypsin. Like MLBK, the activity of the peptide increased about 8-fold when treated with human plasma aminopeptidase or dipeptidyl peptidase I to give BK. Amino acid analysis of the purified peptide derived from kininogen B3.2α was consistent with its being MLBK. The peptic kinin also had the same retention volume as MLBK on an analytical SP-Sephadex C-25 column which separates MLBK, LBK, and BK.

2) Human Urinary Kallikrein. a) Two Catalytic Sites: Since human urinary kallikrein, like other glandular kallikreins, cleaves two dissimilar peptide bonds, Met-Lys and Arg-Ser, in kininogens to form lysyl-bradykinin, we have for several years thought it likely that these kallikreins would have two non-identical catalytic centers. We treated human urinary kallikrein with DFP until all of the arginine esterase (TAME) activity had been destroyed. The DIP-enzyme was unable to release kinin from LMW kininogen II (bradykinin sequence inside the molecule), but was still able to form kinin from LMW kininogen I (bradykinin at the C-terminus). It is therefore practically certain

that human urinary kallikrein has two different active sites. On the other hand, DFP treatment of rat and horse urinary kallikreins, hog pancreatic kallikrein, and human plasma kallikrein destroyed both active sites. Thus, human urinary kallikrein may be unique in lacking a serine residue in site 2, the active center responsible for cleaving the Met-Lys bond.

b) Kinetics: Human urinary kallikrein, like trypsin and several other serine proteinases, shows substrate activation with TAME, but not with BAEE. The kinetics of human urinary kallikreins A and B (see Serial No. NHLI-134) were carefully studied at 25° between 0.15 and 20 mM TAME. When the data were analyzed according to an equation developed for the hydrolysis of TAME by trypsin (Trowbridge et al., Biochemistry 2:843, 1963), an excellent fit was obtained. As with trypsin and TAME, two sets of kinetic parameters were obtained, one between 0.15 and 1.0 mM TAME and the other between 2.0 and 20 mM.

c) Bioassay of Kallikrein in Urine: Incubation of from 10 to 25 µl of untreated urine with about 0.15 mg of kininogen Prep B for 15 min at 37° yields about 150 ng of LBK, an amount readily detected by the isolated guinea pig ileum. Quantitation in TAME units can be achieved by means of an LBK standard and a curve relating a standard human urinary kallikrein to the amount of LBK produced under the above conditions. Those urine samples in which kininases and aminopeptidases are high relative to kallikrein and might therefore interfere with the bioassay by destroying the LBK formed or by converting it to bradykinin, respectively, can be treated at pH 4.4/1 hr/37° to destroy the kininase and with 1,10-phenanthroline to inhibit the aminopeptidase activity. This method is useful for measuring urinary and other kallikreins in very dilute solutions and for checking the usual TAME and BAEE assay methods for determining kallikrein content.

Significance to Biomedical Research and the Program of the Institute: The purification of the components of the plasma kallikrein system is crucial to investigations of its physiological function(s). This system is activated simultaneously with the intrinsic blood coagulation and lysis systems. It now appears that HMW kininogen, which we had previously isolated in highly purified form, is necessary for the activation of Hageman factor, which in turn initiates these three systems. It will therefore be of great importance to study the chemistry of pure HMW kininogens and the mechanism whereby they act.

Proposed Course: HMW Human Kininogens: We plan to do further experiments designed to discover the conditions under which B4 kininogens acquire the capacity to activate Hageman factor and to look for ways to separate the X antigen from the kininogen part.

Human Urinary and Other Kallikreins: Further work will be done on the two active sites of kallikreins, using various active site reagents.

Keyword Descriptors: plasma kallikrein urinary kallikrein kininogen kinins coagulation

Honors and Awards: None

Publications:

Nustad, K., Pierce, J.V., and Vaaje, K.: Synthesis of kallikreins by rat kidney slices. Brit. J. Pharmacol. 53:229-234, 1975.

Project No. Z01 HL 01945-03 HE
1. Hypertension-Endocrine Branch
2. Physiological Chemistry
3. Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Histamine, Prostaglandins and Esterase From Human Lung

Previous Serial Number: NHLI-286(c)

Principal Investigator: Marion E. Webster, Ph.D.

Other Investigators: Hidenobu Takahashi, Ph.D.
Harold H. Newball, M.D.

Cooperating Units: Johns Hopkins University School of Medicine
Baltimore, Maryland

Project Description:

Objectives: The passive sensitization of human lung fragments as first described by Parish (Nature 215:738, 1967) provided a laboratory model for the study of allergic asthma in man and permits investigation of the role of mediators in this immediate type of allergy. The purposes of this study are to identify mediators released from human lung during sensitization; to determine the mechanism of their release; to assess the role of pharmacological compounds used in the treatment of allergy; and to provide a rational basis for the development of new drugs.

Methods Employed: Human lung obtained during surgery is cut into fragments (30-100 mg), sensitized with sera obtained from individuals allergic to ragweed, washed free of serum proteins and incubated with a preparation of pure antigen E (5-500 ng). Release of arginine esterase was determined by sensitive radiochemical assays developed in this laboratory (Beaven et al., Clin. Chim. Acta 32:67, 1971).

Major Findings: An improved method for the measurement of histamine has been developed by combining the procedure of Thithapanda et al. (Comp. Gen. Pharmacol. 3:139, 1972) with that of the method previously employed (Beaven et al., Clin. Chim. Acta 37:91, 1972). This greatly simplified method has been applied routinely to the measurement of the release of histamine in lung supernatants and the results correlate well with the earlier method.

In addition to histamine, an arginine esterase and SRS-A, prostaglandins are also released when passively sensitized human lung is reacted with specific antigen. Measurement of the prostaglandins released by radioimmunoassay

(NHLI-99c) indicated that the increase in prostaglandin F was much smaller than that previously reported (Piper and Walker, Br. J. Pharmacol. 47:291, 1973). Prostaglandin E-like substances, on the other hand, increased in a manner similar to that of histamine and arginine esterase. As prostaglandin F's constrict and prostaglandin E's relax human bronchi, it appears unlikely that prostaglandins have a direct mediator effect in bronchial asthma. However, they may act as modulators of this disease either by potentiating the effects of other mediators or by altering the levels of cyclic AMP.

Our earlier results (Webster et al., Ciencia e Cultura 26:372, 1974) had indicated that the arginine esterase released from human lung by antigen-antibody interaction was neither a kallikrein nor an activator of plasma kallikrein. This esterase is also neither plasmin nor a plasminogen activator. After antigen challenge, the lung incubation medium contained no detectable plasmin (<0.2 ng) and the small amount of plasminogen activator found increased only two-fold under conditions which caused a four-fold or greater increase in histamine and arginine esterase activity.

The arginine esterase activity is found in the precipitate of an 80% ethanol extraction of SRS-A (Slow Reacting Substance of Anaphylaxis) (NHLI-285(c)). When this precipitate was suspended in 0.04 M Tris, pH 8.0, the enzyme was insoluble and could be removed by slow-speed centrifugation. The addition of 3 mM EDTA-2Na solubilized the enzyme suggesting that its insolubility was due to formation of an insoluble salt. The enzyme was stable in 0.25 M Tris pH 5.0 to 8.0, but unstable at pH 4.0 and below losing 50% of its activity in 4 hrs at 4° at pH 4.0 and greater than 90% at pH 3.0. The enzyme also showed unusual lability with various salts. For example, in one-half hour at room temperature it lost 40, 50 and 80% of its activity, respectively, on standing in 0.2 M LiCl, NaCl and KCl dissolved in 0.14 M Tris buffer, pH 8.0.

Initial attempts to purify the arginine esterase by chromatography on DEAE-cellulose were unsuccessful. Although the enzyme readily adsorbed, it could not be removed under the above constrictions of pH and ionic strength. However, the enzyme did adsorb to CM-cellulose at pH 5.0 and could be eluted by applying a linear gradient from 0.05 M Tris-acetate, pH 5.0, to 0.15 M Tris-acetate, pH 8.0. Under these conditions 46% of the activity could be recovered as a single peak, which paralleled the protein concentration. Two additional small peaks of activity were recovered, represent 12 and 8% of the starting activity. Despite the number of purification steps, the arginine esterase recovered in the main peak had only been purified 9-15 fold, suggesting that this esterase may represent a significant portion of the protein released following antigen-antibody interaction. However, further evidence to establish the purity of these preparations will be required.

Significance to Biomedical Research and the Program of the Institute: The identification of mediators released from human lung by antigen-antibody reaction coupled with an investigation of the mechanism of their release and the compounds which inhibit this release, should provide a rationale for the development of therapeutic agents for bronchial asthma.

Proposed Course: Continued investigation into the mediators and modulators released from human lung by antigen-antibody interaction.

Keyword Descriptors: histamine prostaglandins arginine esterase lung bronchial asthma

Honors and Awards: None

Publications:

Maling, H.M., Webster, M.E., Williams, M.A., Saul, W. and Anderson, W., Jr.: Inflammation induced by histamine, serotonin, bradykinin and compound 48/80 in the rat. J. Pharm. Exp. Therap. 191:300-310, 1974.

Miller, R.L., Webster, M.E. and Melmon, K.L.: Interaction of leukocytes andotoxin with the plasmin and kinin systems. J. Europ. Pharm. (In press), 1975.

Oh-ishi, S. and Webster, M.E.: Vascular permeability factors (PF/Nat and PF/Dil): their relationship to Hageman factor and the kallikrein-kinin system. Biochem. Pharm. 24:591-598, 1975.

Oh-ishi, S. and Webster, M.E.: Formation of prekallikrein activator and TAME esterase activity by dilution of human plasma. In Pisano, J.J. (Ed.): Chemistry and Biology of the Kallikrein-Kinin System in Health and Disease. Washington, D.C., U.S. Government Printing Office, 1975, (In press).

Webster, M.E. and Oh-ishi, S.: Activation of Hageman factor (Factor XII): requirement for activators other than prekallikrein. In Pisano, J.J. (Ed.): Chemistry and Biology of the Kallikrein-Kinin System in Health and Disease. Washington, D.C., U.S. Government Printing Office, 1975, (In press).

Project No. Z01 HL 01946-02 HE
1. Hypertension-Endocrine Branch
2. Physiological Chemistry
3. Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Purification of SRS-A from Human Lung

Previous Serial Number: NHLI-285(c)

Principal Investigator: Hidenobu Takahashi, Ph.D.

Other Investigators: Marion E. Webster, Ph.D.
Harold H. Newball, M.D.

Cooperating Units: Johns Hopkins University School of Medicine
Baltimore, Maryland

Project Description:

Objectives: SRS-A (Slow Reacting Substance of Anaphylaxis) is released from passively sensitized human lung by addition of specific antigen and has been proposed as a mediator of bronchial asthma. First described in 1940, a number of attempts have been made to purify this substance from guinea pig, cat and rat tissue. Although much progress has been made, the precise chemical structure of this compound has still not been determined. In the present study we are again attempting to isolate SRS-A and to determine its chemical structure.

Methods Employed: Human lung fragments (30-100 mg) were passively sensitized with ragweed antisera (1:80 dilution), washed free of serum proteins, and incubated for 15-30 min at 37° with specific antigen E (500 ng/tissue piece). The reaction was terminated by removal of the tissue. Biological activity was measured by bioassay using the isolated guinea pig ileum in the presence of atropine and triprolidine.

Major Findings: Previous studies from this laboratory (NHLI-285(c)) had shown that SRS-A from human lung could be separated into four distinct fractions by extraction with ethanol, base hydrolysis (0.1 M NaOH for 30 min at 37°), filtration and/or elution through Amberlite XAD-2 and chromatography on silicic acid. Yields were 50-75% of the starting activity, provided tyramine (500 µg/ml) was used to protect SRS-A from destruction by ultraviolet light. This procedure was similar to that devised by Orange and coworkers (J. Immunol. 110:760, 1973) for the purification of rat SRS-A. However, in their experiments, rat SRS-A appeared to be homogenous since it adsorbed to XAD-2 and eluted from silicic acid only in the final solvent.

Additional studies have shown that SRS-A's can be further purified by chromatography on DEAE-cellulose. The column is similar to that described by Rouser and coworkers (J. Amer. Oil Chenist's Soc. 38:544, 1961) for the separation of lipids except that methanol:ammonium carbonate was used as the final solvent rather than glacial acetic acid, which destroyed the biological activity of the SRS-A's. In the final procedure, the SRS-A's are separated into the same four biologically active fractions by extraction with ethanol, base hydrolysis and chromatography on silicic acid and DEAE-cellulose. This procedure has been applied to a number of preparations of SRS-A from different lungs and results have been reproducible with an overall recovery through all steps of about 50%.

Orange and coworkers (J. Immunol. 113:316, 1974) reported that arylsulfatases (Type H-I, II and III) destroyed the biological activity of rat and human SRS-A. We have found that SRS-A fractions inhibit the activity of type H-I arylsulfatase at pH 4.5, utilizing p-nitrocatechol sulfate as substrate. Under these conditions this arylsulfatase is inhibited by both sodium sulfate and sodium phosphate to the same extent. SRS-A fractions I, II, III and IV inhibited 5, 3, 46 and 78 µg sulfate equivalent per SRS-A unit, respectively. Fractions III and IV still contain appreciable amounts of salts derived from the original Tyrode's solution and these salts may have contributed to the inhibition seen in these fractions. Nevertheless, measurement of inhibition of arylsulfatase during silicic acid and DEAE-cellulose chromatography gave results which correlated well with biological activity. For example, those fractions which inhibited arylsulfatase also contained biological activity to SRS-A. At this pH and at ratios of arylsulfatase units/SRS-A units of 0.3 to 6.0, type H-1 arylsulfatase failed to destroy the biological activity of these SRS-A's except for Fraction I.

These results suggest that human SRS-A's, like the prostaglandins, may represent a family of compounds. Alternatively, human SRS-A's may be bound to compounds which differ in charge and mobility. This latter possibility would appear unlikely since, prior to separation, the crude SRS-A was extracted with ethanol to prevent its binding to protein and hydrolyzed with base to prevent its binding to phospholipids. The additional possibility that the alkali treatment, itself, had produced the multiple forms deserves further consideration, although similar treatment of rat SRS-A did not result in cleavage. Whether SRS-A contains a sulfate group also remains to be established with certainty. Further studies on the chemical structure are indicated.

Significance to Biomedical Research and the Program of the Institute: SRS-A is one of the few remaining mediators of bronchial asthma whose chemical structure remains unknown. The purification of SRS-A and the determination of its structure should greatly aid the development of antagonists for this vasoactive substance which may well be clinically useful.

Proposed Course: Efforts will continue to devise methods for the further purification and chemical characterization of human SRS-A's.

Keyword Descriptors: lung SRS-A bronchial asthma

Honors and Awards: None

Publications:

Webster, M.E., Newball, H.H., Oh-ishi, S., Takahashi, H., Horakova, Z., Atkins, F.L. and Beaven, M.A.: Release of histamine and arginine esterase activity from passively sensitized human lung by ragweed antigen. Ciencia e Cultura 26:372-376, 1974.

Project No. Z01 HL 01947-01 HE
1. Hypertension-Endocrine Branch
2. Physiological Chemistry
3. Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Biosynthesis of SRS-A in Monkey Lung

Previous Serial Number: None

Principal Investigator: Barbara Davis, M.S.

Other Investigators: Marion E. Webster, Ph.D.

Cooperating Units: None

Project Description:

Objectives: Although SRS-A (Slow Reacting Substance of Anaphylaxis) was first described in 1940 by Kellaway and Trethewie and shown to be released from monkey lung in 1966 by Goodfriend et al., (Int. Arch. Allergy 30:511), the biochemical mechanism of its synthesis remains unknowns. The precursors of SRS-A and the enzymes involved in its formation have not yet been described. In this project efforts will be undertaken to study the mechanism of biosynthesis of SRS-A in monkey lung.

Methods Employed: Monkey lung fragments (50-100 mg) or homogenates are sensitized with human ragweed antisera, washed free of serum proteins, and incubated for 5-30 min at 37° with varying concentrations of antigen E (5-5,000 ng). The reaction is stopped by removal of the tissue or by chilling to 4°. The biological activity of SRS-A is determined by bioassay utilizing the isolated guinea pig ileum in the presence of anticholinergic and antihistaminic agents. Histamine and arginine esterase are determined by radiochemical techniques described elsewhere (Webster et al., Ciencia e Cultura 26:372, 1974; project report #Z01 HL 1945-03 HE).

Major Findings: Previous studies in this laboratory demonstrated the release of an arginine esterase, as well as SRS-A and histamine, from passively sensitized human lung on addition of specific antigen. Monkey lung also releases an arginine esterase although at concentrations approximately 1/10 that of human lung.

The amount of antiserum required to passively sensitize monkey lung is similar to that previously found for human lung. With monkey lung, however, higher concentrations of antigen E and/or longer periods of incubation at 37° are required to effect similar release of histamine and/or SRS-A. SRS-A is released from monkey lung at a significantly slower rate than histamine when less than maximal amounts of antigen E are employed, suggesting intra-



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cellular synthesis of SRS-A as opposed to the immediate release of preformed histamine.

As with human lung, the optimal concentration of antigen E required for release varies with each tissue. Preliminary experiments suggest that the release of arginine esterase may correlate with synthesis of SRS-A. In order to select the proper antigen concentration, therefore, each tissue will be tested with varying concentrations of antigen employing the rapid radiochemical method for arginine esterase.

A biochemical method for the detection of SRS-A activity, such as inhibition of arylsulfatase activity (Z01 HL 1946-02 HE), is desirable. Also, studies of SRS-A formation in monkey lung homogenates have been difficult to interpret, since homogenates contain substance(s) other than histamine and SRS-A which contract the guinea pig ileum. In monkey lung, as in human lung, SRS-A occurs in the 80% ethanol extracts. Thin-layer chromatography of these extracts on silicic acid resulted in nearly complete loss of activity even though a nitrogen atmosphere and the absence of light was employed to reduce lability. Present evidence suggests that the salts in the ethanol extracts can be successfully removed by chromatography on Sephadex LH-20 in 80% ethanol (Kuritzky and Goodfriend, Int. Arch. Allergy 46:522, 1974) without major loss of activity. Further studies will be required to determine whether a biochemical method can be developed for measurement of SRS-A activity.

Significance to Biomedical Research and the Program of the Institute: The determination of the mechanism of biosynthesis of SRS-A should assist in the development of antagonists of SRS-A which may be of therapeutic value in bronchial asthma.

Proposed Course: To continue investigation into alternate methods for the determination of SRS-A activity and to initiate studies on the mechanism of biosynthesis of SRS-A in monkey lung.

Keyword Descriptors: SRS-A lung monkey bronchial asthma biosynthesis

Honors and Awards: None

Publications: None

Project No. Z01 HL 01948-03 HE
1. Hypertension-Endocrine Branch
2. Physiological Chemistry
3. Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Role of Prostaglandins in the Vascular System

Previous Serial Number: NHLI-283

Principal Investigator: Lauren M. Cagen, Ph.D.

Other Investigators: John J. Pisano, Ph.D.
Henry M. Fales, Ph.D.
Robert E. Bowden, M.D.
William Jakoby, Ph.D.
Marjorie P. Peyton, B.S.

Cooperating Units: Laboratory of Chemistry, NHLI
Section on Enzyme and Cellular Biochemistry
National Institute of Arthritis, Metabolism and Digestive Diseases

Project Description:

Objectives: To determine the structure of polar forms of prostaglandin formed by exposure of PGA to human red blood cells and to assess the significance of this mode of metabolism in the physiology and pharmacology of PGA.

Methods Employed: Metabolites of PGA were purified by thin-layer and column chromatography and analyzed by gas chromatography and mass spectrometry.

Major Findings: A method for extracting prostaglandins from plasma has been modified to give consistent >85% yields of prostaglandin A. Formerly yields were sometimes as low as 23%. Modifications consisted of, 1) thoroughly washing glassware with methanol, 2) careful control of silicic acid column length, 3) washing column with 2 ml benzene after sample application to remove non-polar lipid material, 4) dissolving the final sample in a small amount of methanol (amounting to 10% of intended final volume) before attempting to take it up to buffer. Using methanol in the final sample necessitates using 10% methanol in the radioimmunoassay. This presented no problem. Straight lines with slopes very near 1.0 were obtained for log-logit plots of standard curves in which all tubes contained 10% methanol. Sensitivity was equal to that obtained when no methanol was used.



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Using this modified method, 1 ml samples of normal plasma were extracted in triplicate and assayed for prostaglandin A. Values of 250±200 pg/ml were obtained. The large error with minute quantities necessitates the testing of larger plasma samples. Extraction of 1 ml water or buffer and radioimmunoassay for PGA consistently gave values of 0.

$PGA_1$ is converted to whole humn blood and by suspensions of human red cells to a polar form(s) without vasodepressor activity. The polar products formed when $^3H$-$PGA_1$ was incubated with tris-saline suspensions of saline washed human red blood cells were purified by solvent extraction and XAD column and cellulose thin-layer chromatography. Combined gas chromatography-mass spectrometry revealed the presence of 2 prostaglandin derivatives in the purified samples. The first of these thermolyzes to $PGA_1$ during gas chromatography; the second compound thermolyzes to a novel prostaglandin derivative which contains 2 hydroxyl groups, no keto group, and the elements of $H_2S$ and which was assigned the structure: 9,15-dihydroxy-11-mercaptoprost-13-enoic acid. This compound was also obtained by treating $PGA_1$ with $H_2S$ and $NaBH_4$ successively.

Amino acid analysis of samples of purified red cell product hydrolyzed in 6 N HCl showed the presence of glutamic acid and glycine in quantities stoichiometric with prostaglandin, and of lesser amounts of cystine. No other amino acids were observed. This strongly implies that the polar red cell products are glutathione conjugates of $PGA_1$, containing one molecule of glutathione per molecule of prostaglandin. NMR of the red cell product showed that the 13,14 double bond was still present while the 10,11 double bond had disappeared. Thus the first of the red cell products results from conjugate addition of GSH to the 11 position of $PGA_1$; the second product arises from the first by enzymatic reduction of the 9-keto group.

GSH reacts rapidly and nonenzymically with $PGA_1$ at physiological pH and 37°. The product of this reaction was identical in chromatographic mobility to the red cell product in all systems of TLC and paper chromatography tested. Samples of synthetic GSH conjugate were reduced with $NaBH_4$. The reduced and unreduced GSH conjugate, and the red cell product all had the same chromatographic mobility.

The reaction of $PGA_1$ with GSH may also be enzymically catalyzed. Four highly purified GSH S transferases were tested for activity towards $PGA_1$; all were found to be active. No difference in the enzymatic and non-enzymatic product was observed.

As expected, the GSH conjugate of $PGA_1$ thermolyzes to $PGA_1$ during gas chromatography, while the $NaBH_4$ reduced GSH conjugate thermolyzes to the same diol mercaptan derivative observed during gas chromatography of red cell product. Thus the synthetic GSH conjugates and the red cell products appear identical.

The same $PGF_1$-like material is liberated by treatment of the reduced GSH conjugate and the polar red cell product with BrCN, further confirming the identity of these 2 substances. However the BrCN fragment appears from its

mass spectrum and from its sensitivity to periodate cleavage to be a glycol-containing rearrangement product of $PGF_1$ rather than $PGF_1$ itself.

In experiments performed in dogs, $^3H$-$PGA_1$ was infused into the renal artery. About 75% of the radioactivity appearing in the urine, (20% of that added) and more than 25% of the radioactivity appearing in the renal venous effluent, 48% of that added, were converted to highly polar forms. Almost no unaltered $PGA_1$ was recovered. In experiments with guinea pigs 35% of the $^3H$-$PGA_1$ injected appeared in the urine after 24 hrs, and almost 100% of this had been converted to polar metabolites. These metabolites formed in dogs and in guinea pigs are more polar than any of the previously described metabolites of $PGA_1$, but are consistent with the high polarity of glutathione conjugates of $PGA_1$.

Samples of human urine were examined for the possible presence of prostaglandins. Since seminal plasma contains high levels of prostaglandins, urine samples from female subjects were employed. Significant levels of immunoreactive $PGB_2$ ($iPGB_2$) were liberated from the aqueous residue of samples of human urine after removal of primary prostaglandins and their less polar metabolites by solvent extraction. Levels of $iPGB_2$ obtained ranged from 140 to 300 pg/ml and corresponded to at least 50% of the $PGE_2$ content of the same urine samples. Since it is not yet possible to estimate the recovery of prostaglandin by this procedure, it is probable that the actual level in urine is considerably higher.

Significance to Biomedical Research and the Program of the Institute: Prostaglandins of the A series have potent vasodilator and natriuretic effects and have been used experimentally in the treatment of hypertension; these compounds are able to maintain normal levels of sodium excretion at the same time that blood pressure is lowered. It has been reported that PGA may be formed *in vivo* and reach physiologically significant levels in human plasma in normal and pathological states.

Little is known about the metabolism of PGA in humans or other animals. We have shown that $PGA_1$ is converted by human red blood cells to $PGA_1$-GSH conjugate. Although $PGA_1$ reacts readily with GSH nonenzymically, this reaction is also catalyzed by enzymes present in high concentration in liver and kidney.

Preliminary experiments indicate that significant amounts of prostaglandin may exist in urine in conjugated form. This is the first report that prostaglandin conjugates form *in vivo*. Although further work is necessary to establish the chemical nature of these conjugates, this result is strongly suggestive that $PGA_2$, a substance known to have potent effects on renal blood flow and diuresis, is formed in the human kidney *in vivo*.

It was found that $^3H$-$PGA_1$ injected into canine renal artery was almost entirely converted into other chemical forms in one passage through the kidney. The ability of the kidney to rapidly metabolize PGA may account for past failures to observe PGA in renal venous effluent after infusion of chemical stimulators of prostaglandin synthesis.



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In the course of this work, it was found that PGA is highly susceptible to Michael-type addition reactions by sulfur nucleophiles. This reactivity is of potential physiological significance, since many enzymes, including such key membrane enzymes as Na-K ATPase and adenyl cyclase, are inhibited by alkylating agents. It is of interest in this regard that PGA shares some of the renal effects of the diuretic agent ethacrynic acid, a substance which also contains an α,β-unsaturated ketone. It is conceivable that some of the biological effects of PGA are due to its ability to alkylate protein sulfhydryl groups.

Proposed Course: The chemical nature of the conjugated forms of prostaglandin found in human urine and of adducts formed from $PGA_1$ by passage through dog kidney will also be determined.

The levels of this substance(s) in urine will be assessed by radioimmunoassay of the $PGB_2$ liberated by treatment with KOH. Levels in normal human female urine will be established and compared with levels in pathological states thought to affect prostaglandin production, e.g., renal artery stenosis, essential hypertension, and malignant tumors.

The natural occurrence of polar conjugates of PG in urine raises the question of the ability of kidney to generate PGA. The ability of kidney of various species including human to convert $PGE_1$ to $PGA_1$ will be assessed, using assays for $PGA_1$ in the conjugated and unconjugated forms.

Bradykinin is known to be a potent stimulator of prostaglandin synthesis in mammalian kidney and in vascular endothelium. It has been suggested that bradykinin acts to stimulate an endogenous lipase which liberates the unsaturated fatty acid precursors of prostaglandins. Experiments to test this hypothesis will be performed, using homogenates of rabbit renal medulla. The effect of bradykinin on unsaturated fatty acid release and on the generation of possible mediators of hormone action, such as cAMP and cGMP will be measured. If preliminary experiments are successful, the characterization of the renal hormone-sensitive lipase will be undertaken.

Keyword Descriptors: prostaglandins glutathione red blood cells urine

Honors and Awards: None

Publications:

Cagen, L.M., Pisano, J.J., and Fales, H.M.: Glutathione adducts of $PGA_1$. Fed. Proc. 34:790, 1975.



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Project No. Z01 HL 01949-02 HE
1. Hypertension-Endocrine Branch
2. Physiological Chemistry
3. Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Amino Acid Sequence Determination of Polypeptides

Previous Serial Number: NHLI-282

Principal Investigator: Henry C. Krutzsch, Ph.D.

Other Investigators: John J. Pisano, Ph.D.
Henry M. Fales, Ph.D.

Cooperating Units: Laboratory of Chemistry, NHLI

Project Description:

Objectives: To continue the successful development of a new method for the determination of the amino acid sequence of polypeptides with emphasis on improvement in scope and sensitivity. The technique utilizes digestion of the polypeptide with dipeptidyl aminopeptidase (DAP), followed by gas chromatography-mass spectrometry (GC-MS) to identify the suitably derivitized dipeptide products.

Methods Employed: The method generally employed in the DAP/GC-MS method of polypeptide sequencing is as follows:

1) Separate DAP digests of the polypeptide and its single Edman degraded cogener.

2) GC-MS of the volatile dipeptide derivative mixtures for separation and identification of the dipeptides.

3) Combination of the two sets of dipeptides to yield the polypeptide structure.

Major Findings: 1) Enzymology: A major thrust has been centered on improving and expanding the enzymology involved in the DAP/GC-MS method of polypeptide sequencing. As a result of this effort, the scope of polypeptides which can be sequenced by this technique has been enlarged. Previously, when DAP-I was the sole enzyme employed, digestion of polypeptides containing proline came to a halt at the point when this moiety appeared one or two residues distal to the amino terminus. Digestion also ceased when a lysine or arginine residue appeared at the amino terminus.



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The proline problem was surmounted by the application of two other more specific DAP enzymes, DAP-IV and V. DAP-IV has the ability to cleave the peptide bond involving the carboxyl group of proline. This enzyme had been previously reported but much more work was required before it could be used successfully in polypeptide sequencing. Efforts were directed at removal of previously unrecognized contaminating proteases, elimination of GC background-forming material and further enzyme characterization, chiefly defining the scope of the enzyme's activity toward various peptide substrates.

DAP-V, a previously unreported DAP enzyme, has the ability to cleave the peptide bond involving the amino group of proline. Thus, the β-naphthylamides of the polypeptides val-ala-pro-ala, gly-pro-pro-ala, pro-ala-pro-pro, and gly-pro-pro-pro were successfully digested. The possibility that these digestions were effected by monopeptidases or endopeptidases was ruled out in other experiments. Final confirmation of the presence of the expected dipeptide products was obtained via GC-MS. Work is presently under way to purify large amounts of this enzyme to establish its usefulness in the sequencing of polypeptides.

The problem with lysine and arginine residues was solved through further investigation of the enzymology of DAP-I. Thus, by further alterations in reaction conditions, chiefly in pH and amounts of activators used, polypeptides containing lysine or arginine at the amino terminal, or in a position allowing them to appear at the amino terminal during digestion, could be digested by DAP-I. These changes caused some GC background, but it was not of any consequence.

Another result of these investigations was to further increase the yield of dipeptides produced from DAP digestion. Coupled with some procedural improvements in dipeptides derivitization, the lower limit of sensitivity has been extended down to the range of 1-2 nanomoles of polypeptides. Previously, 10-20 nanomoles of peptide was required for total sequence analysis.

2) Volatile dipeptide derivatives and gas chromatography: The trimethylsilyl derivative is now the standard volatile dipeptide derivative used. Several GC columns have been tried but the 2 mm x 0.6 m 1% OV-1 column previously described has proven to be the best. One useful finding was that a 2 mm x 0.6 m 1% Dexsil 300 column can be used as a supplemental column giving slightly different resolution characteristics.

3) GC-MS: The number of dipeptides observed has now increased to over 150 out of the 400 possible. This increase has provided at least several representatives of dipeptides containing a given member of the common 20 aminoacids in either the amino terminal or carboxyl terminal position of the dipeptide. This additional information will permit the confident identification of any newly encountered dipeptides. The need for direct MS probe introduction for identification of dipeptides containing His,Gln and Asn has been partially removed through better masking of active sites in the GC, but still remains an absolute requirement for those dipeptides containing arginine. Techniques for direct probe identification of such dipeptides have also been improved by several changes in the experimental procedure employed in this operation.

4) Polypeptide unknowns: Seven more polypeptide unknowns were successfully sequenced. One contained amino terminal arginine, another contained proline. Six of the seven were sequenced in two days.

Significance to Biomedical Research and the Program of the Institute: Greater sensitivity and confidence is required for elucidating the structures of minute quantities of biologically important polypeptides including hormones, neurotransmitters, cell growth factors, transplantation antigens, etc. The investigation described above has further demonstrated and expanded the scope and utility of the promising DAP/GC-MS technique for polypeptide sequence determinations.

Proposed Course: Further work will continue to center on improving the enzymology involved in the DAP/GC-MS sequencing method. In addition, the list of polypeptide unknowns sequenced by the method will be expanded in order to further demonstrate the scope and usefulness of the DAP/GC-MS method in protein sequencing.

Keyword Descriptors: polypeptide sequencing gas chromatography-mass spectroscopy dipeptidyl amino peptidases

Honors and Awards: None

Publications: None

Project No. Z01 HL 01950-01 HE
1. Hypertension-Endocrine Branch
2. Physiological Chemistry
3. Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Fibrinolytic Inhibitors and Vascular Endothelium

Previous Serial Number: None

Principal Investigator: Gilbert M. Wilcox, M.D.

Other Investigators: Valdemar Hial, M.D., Ph.D.
John J. Pisano, Ph.D.
Michael A. Gimbrone, Jr., M.D.

Cooperating Units: Department of Pathology
Peter Bent Brigham Hospital
Harvard Medical School
Boston, Massachusetts

Project Description:

Objectives: To characterize and determine the level of the components of the fibrinolytic system in pure cultures of endothelial cells produced from human veins.

Methods Employed: Endothelial cells (HEC) and smooth muscle cells (SME) were obtained from human umbilical cord veins by collagenase treatment and dissection. Confluent monolayers of endothelial cells and dense subcultures of smooth muscle cells were grown, pooled, washed, resuspended, frozen and stored. Before testing, samples were freeze-dried and resuspended in Tris buffer 0.2 M pH 7.5 to give approximately 8 x $10^6$ cells/ml. The following components of the fibrinolytic system were studied: plasminogen, plasmin, plasminogen activator, inhibitors of plasminogen activation and antiplasmin. The measurements involved testing the samples before and after activation by urokinase trypsin or kaolin. In addition, we investigated the activation of purified plasminogen by urokinase and the recovery of plasmin added to HEC. The samples were assayed by the fibrin plate method or by the radiochemical esterolytic method using $^3$H-acetyl-glycyllysine methyl ester (ACLME) or $^3$H-tosyl arginine methyl ester (TAME).

Major Findings: 1) No plasminogen was detected in the HEC.

2) No plasminogen activator was detected in the HEC when assayed with fibrin plates, however, low AGLME and TAME esterase activity (which could be

due to activator bound to an inhibitor) was detected in HEC. The esterase activity was not increased by kaolin or trypsin but was slightly inhibited by soybean trypsin inhibitor.

3) A potent inhibitor of human urokinase was found in the HEC. Equal volumes of HEC 8 x $10^6$ cells/ml and urokinase (50 CTAU/ml) gave 100% inhibition of the activator (measured by the fibrin plates).

4) No antiplasmin was detected in the HEC preparation by the fibrin plate assay.

Significance to Biomedical Research and the Program of the Institute: Little is known about the nature and source of inhibitors of plasminogen activators in plasma. The present findings point to the endothelial lining as a source. Endothelial cells classically have been viewed as a source of plasminogen activators and the finding of an inhibitor(s) in endothelial cells changes our understanding of the role of the vascular lining in fibrinolysis. It is possible that the fibrinolytic activity of blood vessels is controlled by the level of inhibitor rather than the activator. This new concept could be of great importance in our understanding of the pathogenesis of thrombotic diseases.

Proposed Course: To isolate and characterize the inhibitor of plasminogen activator in HEC.

Keyword Descriptors: human vascular endothelial cells human vascular smooth muscle cells urokinase urokinase inhibition plasmin fibrinolysis coagulation

Honors and Awards: None

Publications: None

Project No. Z01 HL 01951-01 HE
1. Hypertension-Endocrine Branch
2. Physiological Chemistry
3. Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Angiotensin Converting Enzymes (ACE) in Endothelial Cells

Previous Serial Number: None

Principal Investigator: Valdemar Hial, M.D., Ph.D.

Other Investigators: Gilbert M. Wilcox, M.D.
John J. Pisano, Ph.D.
Michael A. Gimbrone, Jr., M.D.

Cooperating Units: Department of Pathology
Peter Bent Brigham Hospital
Harvard Medical School
Boston, Massachusetts

Project Description:

Objectives: To determine the ACE and kininase activity in pure cultures of human vascular endothelial cells and human smooth muscle cells.

Methods Employed: Human endothelial cells (HEC) and smooth muscle cells (SME) were obtained from human umbilical cords by collagenase treatment and dissection. Confluent monolayers of endothelial cells and dense subcultures of SME were grown, harvested, pooled, washed, resuspended and frozen. They were subsequently freeze-dried and resuspended in Tris buffer 0.2 M pH 7.5 or borate-phosphate buffer .05 M pH 8.0 containing approximately 8 x $10^6$ cells/ml. Aliquots of 10-50 µl of these cells were incubated with pure AI or bradykinin at 37°C pH 8.0 x 5-60 min; control samples were incubated with buffer alone. The effect of chloride and cobalt ions and various inhibitors of ACE and kininase were studied. The amount of angiotensin I or II was assayed on the rat uterus and rat blood pressure. Bradykinin was assayed on guinea pig ileum and dog blood pressure. An increase in AII or decrease in bradykinin during an incubation would be indicative of ACE or kininase activity respectively.

Major Findings: 1) Both HEC and SMC contain AEC and kininases. The SMC contain relatively less of each enzyme compared to HEC.

-) The inhibitory characteristics of several substances on ACE from HEC are summarized in the following table:

| Substance | HEC ACE | HEC Kininase | SMC ACE | SMC Kininase |
|---|---|---|---|---|
| | Inhibition* | | | |
| Absence of chloride | 0 | NT | NT | NT |
| Cobalt ($Co^{++}$) (1 mM) | 0 | NT | NT | NT |
| EDTA (1 mM) | 0 | NT | NT | NT |
| BPF (1 mM) | 0 | $\pm$ | NT | $\pm$ |
| 8-HOQ (4 mM) | 0 | $\pm$ | NT | $\pm$ |
| Mercaptoethanol (1 mM) | 0 | NT | NT | NT |
| SBTI (0.05 mg/ml) | + | NT | + | NT |

* + = 90-100% inhibition
$\pm$ = 20-50% inhibition
0 = no effect
NT = not tested

3) This enzyme releases active products, directly from tetradecapeptide (renin substrate) and this reaction is not inhibited by pepstatin, a potent inhibitor of renin.

Abbreviations used: BPF - Bothrops Potentiator Factor; 8-HOQ - 8-hydroxyquinoline; SBTI - soybean trypsin inhibitor.

Significance to Biomedical Research and the Program of the Institute: It is well established that the renin-angiotensin system is involved in regulation of blood pressure, and that angiotensin converting enzyme is a central component of this system. The present study shows that vascular endothelium is a rich source of ACE. The vessel wall (smooth muscle) also has activity. The enzyme contained in these locations appears to be different from that isolated from lung or kidney by other investigators (based on inhibition characteristics). Since this ACE is present in the vessel lining and wall, it may be involved in the local production of AII which could regulate the tone of the vessels and ultimately blood pressure. There is the possibility

that this ACE is identical to tonin, an enzyme able to split the tetradecapeptide (renin substrate) releasing AII directly since tonin is inhibited by SETI, does not require chloride or cobalt and is not inhibited by BPF, EDTA and mercaptoethanol-characteristics shared by our converting enzyme. These cell cultures provide a unique opportunity to investigate ACE's and kininases for the following reasons: (a) the cultures are pure, allowing a separation of the component parts of the vascular wall not before possible; (b) the cell types are widely distributed in the body and results would effect interpretation of enzyme activities found in any organ by previous investigators; (c) all conclusions are applicable to humans.

Proposed Course: Purification and characterization of the pure enzyme. To study the possible physiological role of the enzyme in blood pressure and arterial tone regulation.

Keyword Descriptors: human vascular endothelial cells human vascular smooth muscle cells urokinase urokinase inhibitor plasmin fibrinolysis coagulation

Honors and Awards: None

Publications: None

Project No. Z01 HL 01981-13 HE
1. Hypertension-Endocrine Branch
2. Section on Neuroendocrinology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Taste and Olfaction

Previous Serial No.: NHLI-106(c)

Principal Investigator: Robert I. Henkin, M.D., Ph.D.

Other Investigators: Clark Lum, Ph.D.
A.L. Larson, M.D.
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Cooperating Units: Campbell Institute for Food Research, NIAMDD, NIAID, NCI, Department of Biochemistry, Washington University, St. Louis, Missouri

Project Description:

Objectives: To investigate in a systematic manner the anatomical, physiological, pharmacological, pathological and chemical correlates of taste and olfaction.

Major Findings: Taste

Anatomy.

1. Histochemical investigations of the mechanisms of taste function. The distribution of acetylcholinesterase (AChE) in the taste bud of the rat circumvallate papilla was investigated by histochemical electron microscopy. Previous reports from this and other laboratories of specific AChE activity around subgemmal and intragemmal nerves and between some taste bud cells were confirmed. In addition dense precipitation of AChE between microvilli in the taste bud pore was observed. These studies suggest that acetylcholine may be involved in some of the early events in the taste process which are believed to occur in the pore area. This hypothesis is based upon several previous observations. (1) There are neurosecretory granules in Type I cells of the taste bud, granules which may represent storage vesicles of acetylcholine; this hypothesis has not yet been documented by systematic investigation, (2) many more cholinergic fibers than adrenergic fibers innervate the taste bud in rat and in man, (3) in Type I familial dysautonomia, in which taste buds are not present, parenteral administration of methacholine restores taste function to or toward normal, (4) $I^{125}$ α-bungarotoxin appears to bind to the microvilli of taste bud membranes suggesting the presence of an acetylcholine receptor. The most likely anatomical candidate for this receptor is the Type III cell of the bud which is the only cell in which neural connections appear to be directed from the bud



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to the brain.

2. Evidence of a contractile mechanism in the taste bud of the mouse fungiform papilla. By means of a epimicroscope, a television screen and a video tape recorder, the pore of the taste buds of fungiform papillae of anesthetized mice were observed directly and recorded. After exposure to the vapor from over a high concentration of HCl the extrusion of fluid through the pore of the taste buds was observed. This extrusion occurred within seconds after the exposure to the HCl vapor. The fluid then receded leaving a ring of material. By electron microscopy the pore was seen to be filled with small vesicles which communicated with the oral cavity. The possible contractile mechanism by which this process occurred was suggested by the observation of a long bundle of tonofilaments in Type II cells of the taste bud. The retraction and subsequent extension of this bundle could account for these observations.

3. Investigations of the architecture of the taste buds of human fungiform and circumvallate papillae. Taste buds from human fungiform papillae have not been previously studied in any systematic manner. Taste buds in fungiform papillae open directly into the oral cavity; they are narrower and somewhat more elongated than taste buds from circumvallate papillae. Taste buds in human fungiform papillae number between 0-10 and usually appear in multiples. Taste buds in human circumvallate papillae lie in crypts, do not directly open to the oral cavity and number between 20-100. The three major cell types previously observed in taste buds from circumvallate papillae have also been observed in each of these taste buds; however, the dense core vesicles seen in Type I cells of taste buds from circumvallate papillae are absent in Type I cells of taste buds from fungiform papillae. Also absent is the dense extra cellular material normally found in taste buds from circumvallate papillae. Functionally, taste buds in human fungiform papillae subserve all taste qualities but are most sensitive to salt and sweet tastants. Taste buds in human circumvallate papillae also subserve all taste qualities but are most sensitive only to sweet tastants.

Chemistry:

1. Purification and properties of miraculin, a glycoprotein from *Synsepalum dulcificum* which provokes sweetness and blocks sourness. In the annual report from this section, 1972-73, the process of purification of miraculin developed in this laboratory was elucidated. Of further note in this regard is the following:

(1) The sugar composition of this glycoprotein, confirmed by thin layer chromatography and by gas liquid chromatography, is xylose, fucose, galactose and mannose which differs significantly from that reported by other groups.

(2) These sugars number 15 per mole of protein and appear in the ratio of (1:1:1:2) for fucose, xylose, galactose and mannose, respectively.

(3) The amino acid composition of native miraculin was determined on 3 samples of the protein on 3 occasions after 24,48 and 72 hours of hydrolysis. These results differ from those previously published by another investigator whose results were obtained from one 24 hour hydrolysis.

(4) The action of miraculin in man differs from that suggested by several investigators but, in part, was similar to that noted by Dr. Linda Bartoshuk. Oral application of miraculin for 1-3 minutes makes all sour tastants taste sweet although the miraculin itself is tasteless. Miraculin also blocks the taste of

sour itself, all sour tastants tasting less sour. A similar blocking effect for bitter tastants has also been observed.

(5) The locus of this effect appears to be the palatal taste buds for the effect of miraculin on the tongue alone was minimal whereas the palatal taste buds (which primarily subserve the sour and bitter taste) participated in this reaction.

(6) An intact system for recognizing the taste of sweet was important for this effect to occur. In patients with aglycogeusia and application of miraculin blocked sourness and bitterness but did not produce any sensation of sweetness.

(7) Further modification of the purification process of miraculin has yielded a significant improvement in purity of the glycoprotein. The initial chromatography on Biogel CM 30 has been modified such that the elution process is carried out with 0.1 M sodium phosphate buffer with a linear pH gradient of 6.5 to 7.2. Previously the gradient extended to 7.8 and much non-specific material was eluted at the higher pH. The purified elute was then lyophillized and dialyzed against 0.1 M carbonate buffer which also resulted in the removal of some additional non-specific material. The resultant material was then placed on a QAE Sephadex A-50 column at pH 9.0 (previously 10.5) and eluted with a linear NaCl gradient from 0.05 - 0.3 M (previously 0.05 - 0.65 M). The eluate from this column yielded constant specific activity as indicated by the constancy of several indicators of protein concentration over the eluted peak.

(8) By these modifications miraculin was eluted as a single peak which was homogeneous on disc gel electrophoresis in SDS buffer and by equilibrium dialysis sedimentation. On isolectric focusing however 7 subunits were observed between pH 3-8.5, the major active subunit observed at pH 8.02. It had a UV maximum at 278 nm and a minimum at 250 nm. The maximum fluorescence emission peaks were at 250 nm and 350 nm with excitation at 280 nm in 0.1 M carbonate buffer at pH 9.0 or 0.1 M phosphate buffer at pH 7.0. The sedimentation constant of miraculin in 0.1 M phosphate buffer was 2.80.

(9) Miraculin apparently binds to taste bud membranes for its sweetness provoking and sourness blocking effects are not altered by 5 M urea but are obviated following rinsing the mouth with a 0.1% solution of SDS.

2. Further work on the modification of miraculin by the action of sodium periodate ($NaIO_4$) and sodium borohydride ($NaBH_4$). After treatment of miraculin with low concentrations of $NaIO_4$ ($3 \times 10^{-6}$M) only galactose was selectively oxidized. Following this procedure the blocking effect of miraculin was preserved, i.e., the sourness of 0.02 M citric acid was reduced, but no sweetness provoking effect was observed. At higher concentrations of $NaIO_4$ the oxidation of the other sugars occurred with xylose being affected next readily (after galactose as noted), then mannose and finally fucose. These studies suggest that (a) some specific sequence of sugars may be important in forming a code by which binding of miraculin to the receptor occurs and (b) that galactose binding to the taste bud membrane may play a special role in the sweetness provoking effect of the glycoprotein.

Analyses of the polysaccharide moeities from miraculin treated with $NaBH_4$ and $^3H_4NaB$ indicated that the fucose moeity was reduced to fucitol. This suggested an unusual oligosaccharide linkage in this glycoprotein. The sweetness provoking activity of miraculin was enhanced 2-3 fold by this procedure and may relate to the reduction of fucose by $NaBH_4$ at a concentration of $10^{-4}$M. These observations further confirm the suggestion that the sweetness provoking effect of miraculin may relate to the nature of its polysaccharide moeities and to their molecular arrangement.

3. Demonstration of non-specific and specific binding of sugars and mouse 2.5 S nerve growth factor to bovine taste bud membranes. Taste bud membranes were isolated from taste buds from bovine circumvallate papillae, and non-taste bud bearing epithelial tissue membranes were isolated from the epithelium surrounding these papillae by techniques previously described. Both tissues were evaluated with respect to their ability to bind various sugars under several conditions.

Non-specific and specific binding of $^{14}C$ labelled sucrose, fructose, glucose, saccharin and cyclamate to taste bud and non-taste bud bearing membranes was studied. For non-specific binding, glucose, fructose and sucrose exhibited greater sugar binding capacity to the taste bud membrane fraction $P_{4(B)}$ than did the corresponding $P_{2(B)}$ and $P_{3(B)}$ fractions. For non-taste bud membrane fractions, there was no difference in sugar binding capacity to any of these membrane fractions (Table I). Binding to any purified fraction obtained from taste bud membranes was greater than that for the original filtrate. For the membranes obtained from the epithelial tissue no increase in relative specific activity over that of the original filtrate was observed.

For specific or competitive binding, labelled sucrose, fructose, glucose, cyclamate and saccharine bound specifically to taste bud membranes but only non-specifically to corresponding non-taste bud bearing membranes. Labelled lactose bound non-specifically to both taste bud and non-taste bud membranes and no specific binding could be obtained.

Binding of labelled sugar to taste bud membranes was both dose and temperature dependent, and was inhibited by increasing concentrations of unlabelled sugar, EDTA (79%), NaCl (54%), neuraminidase (40%), phospholipase A (38%), and urea (30%). Dissociation constants were in the range of $10^{-3}$M, consistent with the preference thresholds in cow (sucrose > fructose > glucose; lactose no response). High dissociation of constants for saccharine and cyclamate are consistent with the lack of preference responses to these substances in cow.

Using bovine taste bud membranes obtained from the $P_{4(B)}$ fraction $I^{125}$ 2.5 S mouse nerve growth factor (NGF) exhibited dose dependent binding. Specific displacement of $I^{125}$ NGF by native NGF was also observed.

TABLE I

| BUD FRACTION | $^{14}$[C]-GLUCOSE | $^{14}$[C]-FRUCTOSE | $^{14}$[C]-SUCROSE |
|---|---|---|---|
| | ( x $10^{-4}$ cpm/mg Protein) | | |
| Filtrate | 3.68 (1) | 15.30 (1) | 3.64 (1) |
| $P_{2(B)}$ | 11.98 (3.2) | 56.90 (3.7) | 11.27 (3.1) |
| $P_{3(B)}$ | 35.91 (9.7) | 130.00 (8.5) | 25.54 (7.0) |
| $P_{4(B)}$ | 34.84 (9.4) | 272.50 (17.8) | 86.76 (23.8) |
| $GP_1$ FRACTION | $^{14}$[C]-GLUCOSE | $^{14}$[C]-FRUCTOSE | $^{14}$[C]-SUCROSE |
| | ( x $10^{-5}$ cpm/mg Protein) | | |
| Filtrate | 2.23 (1) | 8.81 (1) | 3.33 (1) |
| $P_{2(B)}$ | 2.92 (1.3) | 9.47 (1.1) | 3.66 (1.1) |
| $P_{3(B)}$ | -- | -- | -- |
| $P_{4(B)}$ | 1.74 (.8) | 7.81 (.9) | 1.78 (.5) |

()Relative specific activity

4. Isolation, purification and determination of some of the chemical characteristics of gustin, a zinc containing protein obtained from human parotid saliva. In my annual report 1972-73 the existence of a zinc containing protein in saliva was hypothesized. Work during this year has resulted in the isolation, purification and determination of some of the chemical characteristics of this protein.

The basis for this hypothesis was based upon several physiological observations. Saliva plays an important role in taste. Patients with xerostomia exhibit hypogeusia and associated pathological changes in taste bud structure. Similar observations of adverse changes in taste acuity have been made in desalivate rats. Oral administration of various non-protein electrolyte solutions has been unsuccessful in restoring taste function to normal in patients with xerostomia whereas treatment of these patients with systemic agents which restore salivary function to normal has been associated with the recovery of normal taste acuity and the appearance of normal taste buds. Zinc is another important factor in taste perception. Patients with hypogeusia of various etiologies show pathological changes in taste buds which are similar to those observed in patients with xerostomia. Patients with hypogeusia in whom salivary flow rates are normal exhibit lower than normal concentrations of zinc in serum and in parotid saliva. Oral administration of zinc to some of the patients with hypogeusia has resulted in the normalization of serum and parotid salivary zinc concentration, taste bud anatomy and taste perception. From these studies and observations we hypothesized that a zinc containing protein was a normal constituent of parotid saliva and its function was, in some manner, related to the growth, nutrition and function to taste buds. In order to verify these hypotheses a zinc containing protein in parotid saliva was isolated, purified and characterized.

Whole parotid saliva was collected from 6 men and 3 women with normal taste acuity. Saliva was lyophylized, dissolved in zinc free water and centrifuged at 20,000 x g for 30 minutes, the clear supernatant fluid used for all subsequent assays.

Gel filtration chromatography was carried out in Sephadex G 150 and DEAE-A50 and in carboxymethylcellulose columns. The relative concentration of protein in parotid saliva was measured by three spectroscopic methods which reflect distinctive protein properties. The absorption at 280 nm is accounted for by the amount of tyrosine and tryptophan in the protein. The intensity of fluorescence at 340 nm is dependent upon the tryptophan quantum yield of the protein. The absorption at 215 nm is determined principally by the peptide chromophore although minor differences will arise if aromatic chromophores are present in unusual amounts. We have used the difference in absorption between 215 and 225 nm since this method largely eliminates any absorbance due to turbidity. Zinc was measured by flameless atomic absorption spectrophotometry. Polyacrylamide gel electrophoreses of each column elute were also performed as was amino acid analysis. Molecular weight determinations by equilibrium sedimentation was performed after the protein was thoroughly dialyzed against 0.1 M NaCl or 6 M guanidine hydrochloride.

(I.) Chromatography

A: Sephadex G 150. Elution with 0.01 M $PO_4$ buffer pH 6.8 produced profiles using each of the three methods of protein determination which are quite distinct since different protein properties are measured by each technique (Fig. 1). The 215 nm profile was divided into five major fractions which were labelled II to VI. An additional fraction, referred to as I, was evident in the solvent front which contained very little 215 nm absorption but was highly fluorescent.

B: DEAE-A50. Fraction II was divided into two parts since the protein in the first part (IIA) contained a much higher Zn/215 nm ratio than did the remaining tubes (IIB). Fraction IIA were lyophilized, dissolved in zinc free water, dialyzed, placed on a DEAE-A50 column, and initially eluted with 0.01 M $PO_4$ buffer pH 6.8 followed by elution with a linear gradient of NaCl (0-0.5 M) in the same buffer. A major separation of the 215 nm absorption peak from the zinc, fluoresence and 280 nm absorption peaks occurred during elution with phosphate buffer. Most of the zinc appeared in two neighboring peaks which were eluted before the NaCl gradient. Moreover the zinc/280 nm ratio was very similar in these two peaks suggesting that the proteins resembled each other rather closely.

C: CMC. The two major and one minor zinc peaks obtained from the DEAE-A50 column were pooled and lyophilized. The product was dissolved in water, dialyzed, placed on a CMC column, and eluted with the same 0.005 M $PO_4$ buffer, pH 5.9 in a linear gradient of NaCl (0-0.30 M). The zinc was largely concentrated in a single peak and the ratio of zinc to protein in this peak was constant and independent of the parameter used to evaluate protein concentration. In the absence of two proteins having all four identical properties the protein in the eluted peak must represent a single molecular species.

The increase in specific activity of the zinc protein (the $Zinc/OD_{215}$; and $Zinc/OD_{280}$), as obtained from the maximum value in the zinc peaks in the three chromatographic columns, is shown in Table II. It is evident that the elimination of the contaminating 280 nm absorbing material is accomplished mainly on the Sephadex G 150 column whereas the elimination of the contaminating 215 nm material occurs mainly on DEAE-A50. There is obviously a great disparity in the 215/280 ratio of many of the proteins present in saliva. The value used to calculate the specific activity of the starting solution was the zinc and 215 nm absorption of whole saliva. Yields were necessarily low since the yield was sacrificed for purity in selecting the most active fraction for further purification on the DEAE-A50 and CMC columns. These studies suggest that a 200-fold purification from the whole parotid saliva was accomplished by these procedures.

(II) Molecular Characterization

A: Polyacrylamide gel electrophoreses. Electrophoresis of the purified human parotid zinc protein revealed one broad band. Electrophoresis in 0.1 M sodium phosphate with 0.1% SDS showed one major band and one faint, more slowly migrating band. After reduction in 2-mercaptoethanol no significant change was noted in the migration of the major band although some diffuse staining was evident

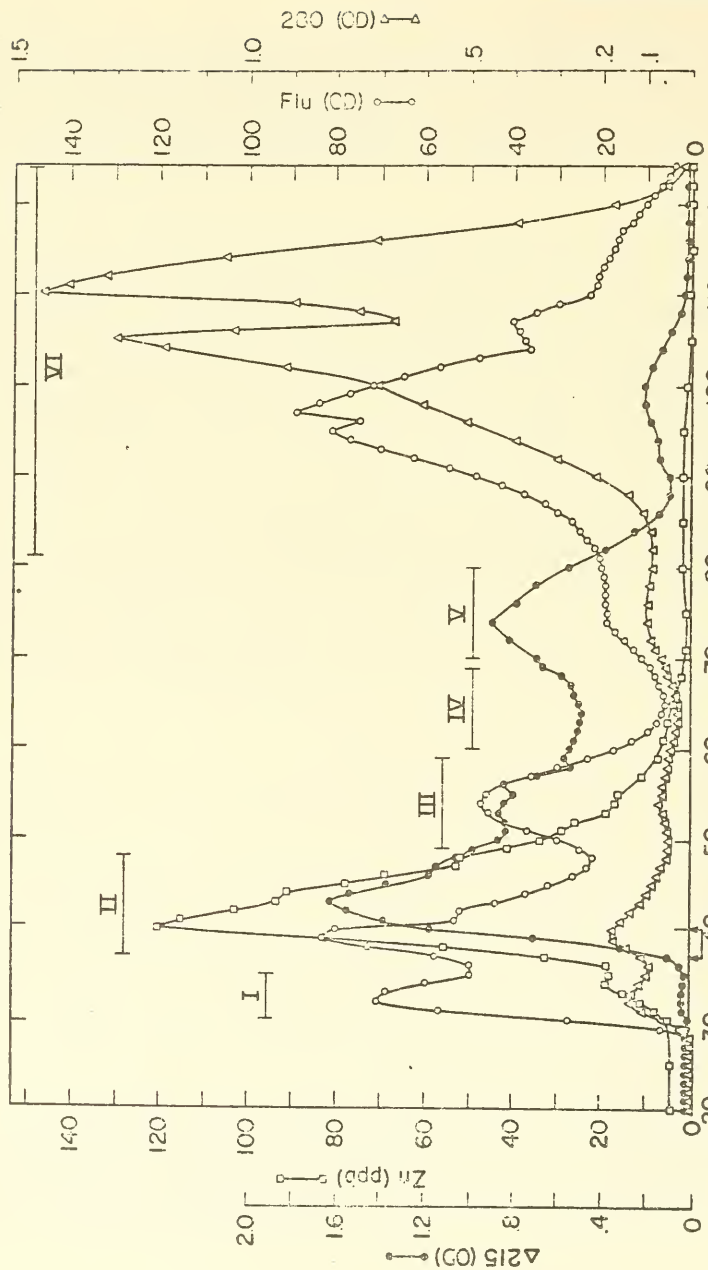


Figure 1.

adjacent to this major band. The molecular weights of the major and minor components in the SDS gels determined by comparison with appropriate standards, were 44,000 and 84,000, respectively.

TABLE II

SPECIFIC ACTIVITY AND YIELD OF PAROTID ZINC PROTEIN

| Substance | Specific Activity Zn/OD 215 ppb/OD | Specific Activity Zn/OD 280 ppb/OD | Yield of Parotid Zn Protein % | Recovery of Total Protein % |
|---|---|---|---|---|
| Whole parotid saliva | 0.05 | 0.19 | -- | -- |
| Sephadex G 150 | 0.58 | 678 | 5.9 | 95 |
| DEAE-A50 | 65.5 | 850 | 6.5 | 84 |
| CMC | 268 | 1040 | 16.5 | 100 |

B: Sedimentation equilibrium. The molecular weight of the purified salivary zinc protein determined by sedimentation equilibrium was 37,000 as calculated from the Svedberg equation. Equilibrium centrifugation in 6.0 M guanidine hydrochloride gave a molecular weight of 35,000 indicating that this molecule does not dissociate into equal subunits in the presence of a reducing agent.

C: Amino acid composition. The amino acid analysis of the purified parotid zinc protein was determined. The histidine content of the parotid zinc protein is high (8 % per mole) while the half-cysteine content is quite low.

D: Zinc-protein ratios. Based upon a molecular weight of 37,000 present estimates suggest that there is two molesof zinc per mole of protein.

PHYSIOLOGY

1. Salt preference and blood pressure response in male rats given estrogen, progesterone and estrogen and progesterone. Preference for various concentrations of Na and K salts and other tastants was determined in 36 normal Sprague-Dawley rats in which (A) estrogen, (B) progesterone, (C) estrogen and progesterone or (D) vehicle alone was administered parenterally, daily for periods of 12 weeks. Weight, food intake and blood pressure were measured at least weekly. Results indicated that on estrogen alone male rats exhibited significantly greater preference for NaCl, $HaHCO_3$, NaAcetate (NaAc) and KCl than did control rats given vehicle alone. This change occurred within two weeks of estrogen administration. However, both estrogen treated and control rats rejected HCl and quinine to a similar degree. Rats given progesterone alone exhibited a significantly greater preference for NaCl and KCl than did controls only after prolonged administration of this hormone and both groups rejected HCl and quinine to a similar degree.

Rats given estrogen and progesterone together exhibited an enhancement over those given either estrogen or progesterone alone in their preference for NaCl, $NaHCO_3$, NaAc, and KCl. These effects occurred prior to those noted for rats given estrogen alone; however, these rats also rejected HCl and quinine to a similar degree as the control rats. No change in blood pressure was observed in any of these rats over the course of the study.

2. Salt taste in patients with essential hypertension and with hypertension due to primary hyperaldosteronism. It is widely believed that excessive salt intake plays a role in the development of hypertension in man. Conversely, it has been demonstrated that deprivation of salt and water is efficacious in the treatment of hypertension. Since salt intake in man is under voluntary control several investigators have measured salt taste acuity in patients with hypertension in an attempt to evaluate the possible role this variable might play in the development of this disease. Their results suggested that, as a group, patients with hypertension exhibited lower than normal salt taste acuity. From this observation one group reasoned that these patients used excessive amounts of salt on their food to achieve the preferred salty taste and thereby unmasked whatever genetic propensity they might have had to develop hypertension.

Although this reasoning is quite attractive critical studies of salt taste acuity in patients with hypertension have not yet been carried out. In an effort to do this specific measurements of salt taste acuity, i.e., detection and recognition thresholds for NaCl, were evaluated in 50 patients with well documented essential hypertension and in 10 patients with hypertension due to primary hyperaldosteronism. In some patients thresholds were measured before and after therapy which was efficacious in lowering blood pressure to or toward normal. Results of these studies indicate that, as a group, taste thresholds for NaCl in patients with either essential hypertension or hypertension due to primary hyperaldosteronism do not differ from normal.

3. Salt preference in patients with untreated and treated essential hypertension. Sodium chloride preference was studied in 16 patients with essential hypertension and 26 normotensive volunteers over a 2 day period. Each exhibited normal detection and recognition thresholds for the taste of NaCl. Each was placed on a constant dry diet containing 9 mEq $Na^+$ and, as the only source of fluid, given a choice of drinking either distilled water, 0.15 M NaCl, or some combination of the two fluids. Patients with essential hypertension drank a significantly greater proportion of their total fluid as saline (day 1: 34.9% versus 12.6%; day 2: 34.1% versus 13.5%) and drank a greater total volume of fluid (day 1: 1332 versus 669 cc; day 2: 1419 versus 824 cc) than did the normotensive volunteers. The total amount of $Na^+$ consumed by the patients was 4.8 to 7.3 times greater than that of the normotensive volunteers. Effective treatment of hypertension lowered mean NaCl preference, $Na^+$ intake and total fluid intake in the four subjects studied under these conditions. These latter findings suggest that treatment with antihypertensive agents may play some role in altering salt appetite.

4. Effects of various orally placed ganglionic blocking agents on taste acuity in man. In order to investigate the neuropharmacology of taste in man various drugs were placed into the oral cavity and taste acuity was measured by determination of taste detection and recognition thresholds and of forced scaling. Drugs included succinylcholine chloride (10,20 and 40 mg) methylcholine chloride (20 mg) curare (9 mg), gallamine (9 mg), reserpine (5 mg) and propranolol (50 mg). Each drug was placed into the oral cavity for 2-3 minutes and then expectorated. Measurements of taste acuity were determined immediately prior to oral placement of each drug and for varying time periods after expectoration. Results indicated that succinylcholine, methacholine and curare significantly inhibited taste acuity for all tastants. Effects of curare persisted for up to 18 hours whereas the effects of succinylcholine and methacholine lasted over a period of one to two hours. Gallamine inhibited taste acuity for bitter and sour tastants and altered forced scaling somewhat for each tastant.

PATHOPHYSIOLOGY

1. Localization of $99^m$ technetium in the region of the nose in Sjögren's syndrome. Patients with Sjögren's syndrome accumulated abnormal amounts of $99^m$ technetium pertechnitate in the region of the nose during isotopic salivary flow studies. It was concurrently and independently observed that many patients with Sjögren's syndrome had hyposmia and pathological changes in their nasal mucous membranes. Fourteen patients with Sjögren's syndrome were studied for the relationship of the above observations and the nasal accumulation of radionuclide was compared with a control group of 16 subjects. Eleven of the 14 patients with Sjögren's syndrome (78%) has nasal accumulation of the radionuclide; 14 had hyposmia and 13 of 14 had chronic inflammation of the nasal mucous membrane. One of the 16 controls (6%) localized radionuclide in the nasal region. Results suggest that hyposmia, inflammatory changes in the nasal mucous membrane and nasal accumulation of $99^m$ technetium pertechnitate are interrelated aspects of Sjögren's syndrome. This nasal accumulation of radionuclide has been termed Rudolph Sign.

2. Nasal mucous membrane biopsy in Sjögren's syndrome: A new diagnostic technique. A procedure was developed for biopsy and light microscopic evaluation of the nasal mucous membrane. This procedure was applied in 15 female patients with Sjögren's syndrome and the results compared with those of a similar procedure applied to the lip. Results indicated that the pathological changes found in the nose were similar to those found in the lip. The most characteristic pathological feature in the nose and in the lip was periglandular infiltration by chronic inflammatory cells with glandular atrophy. These changes in the nose have been compared with similar biopsies obtained in 160 other female patients with hyposmia of varying etiologies and these changes were observed only in patients with Sjögren's syndrome. Biopsy of the nasal mucous membrane is a simple technique which can be employed repeatedly and has little morbidity. As such, it is a useful technique to aid in the diagnosis and evaluation of therapy of Sjögren's syndrome and in the differential diagnosis of hyposmia.

3. Abnormalities of taste and smell after head trauma. Abnormalities of taste and smell were studied in 29 patients after head trauma. These abnormalities included decreased taste acuity (hypogeusia), a distortion of taste acuity (dysgeusia), decreased smell acuity (hyposmia), and a distortion of smell acuity (dysosmia). This syndrome can occur even after minimal head trauma and can begin months after the moment of injury. The patients exhibited a significant decrease in total serum zinc concentration (patients, 77 ± 3 μg/100 ml, mean ± 1 SEM, vs controls, 99 ± 2 μg/100 ml, $P > 0.001$) and a significant increase in total serum copper concentrations (113 ± 4 μg/100 ml vs 100 ± 2 μg/100 $P < 0.001$) compared with control subjects. Mean salivary zinc levels in these patients are also significantly lower than in normal subjects. Symptoms of hypogeusia, dysgeusia, and dysosmia are frequent sequelae of head injury and are important to the patients and to their care after trauma.

4. Clinical aspects of post influenzal hypogeusia, dysgeusia, hyposmia and dysosmia. We have recently described a syndrome characterized by hypogeusia, with or without dysgeusia, and hyposmia, with or without dysosmia. Eighty-seven of 143 consecutive patients studied at the Taste and Smell Clinic at the NIH developed the above symptoms following an influenza-like illness a clinical entity which we have named post influenzal hypogeusia and hyposmia (PIHH). The clinical findings of these 87 patients were defined and related to specific pathophysiological changes.

Patients ranged in age from 19-83 years (mean, 54 years), were of both sexes, of varied ethnic and racial groups and residents of various geographical regions in the world. In each subject the onset of PIHH followed an upper respiratory illness which was clinically of viral origin and usually occurred in the winter or spring months. The illness began as a severe coryza with fever, chills, malaise, anorexia, arthralgia and myalgia. Lower respiratory and gastrointestinal symptoms were occasionally present but not prominent. At the onset of the coryza the patients usually noted an abrupt decrease in taste acuity (hypogeusia) and smell acuity (hyposmia). At first, most patients were not overly concerned about these changes since many had experienced similar hypogeusia and hyposmia with previous influenza-like illnesses. However, during the recovery period, they noted that their taste and smell acuity did not return to normal. Approximately one-half of the patients also developed symptoms of dysgeusia and dysosmia. These symptoms usually began during the recovery period. In about one-half of these latter patients the dysgeusia and dysosmia became increasingly severe with time whereas in the other half they fluctuated in intensity, usually decreasing and disappearing over the next six months.

Following the influenza and the development of PIHH patients noted dryness of their nasal passages and decreased frequency and quantity of nasal discharge. Neither nasal crusting nor epistasis was prominent and nasal stuffiness was uncommon. There was no associated decrease in tearing, salivary flow or secretion from other glands. Patients never complained of any associated oral ulcerations, gingivitis, "cold" or "canker" sores. Swelling of the parotid or other salivary glands was never evident and lymphadenopathy in the head and neck region was not a common feature either during the acute or recovery phase of the influenza.

Patients were referred to our clinic by physicians who had exhausted all their knowledge of diagnosis or treatment of these distressing symptoms. Patient were referred from local family physicians, neurologists, otorhinolaryngolosists and psychiatrists.

Physical examination of the head and neck:

Patients were examined 3 months to 10 years following their initial illness. The most prominent consistent clinical feature of PIHH was in the nose where the tenacious, clear, occasionally white mucous blanket normally found was not observed. The nasal mucous membranes were moist but neither shiny nor glistening. The membranes were often pale in color, but not similar to the pale, boggy bluish tinge commonly observed in allergic rhinitis or the reddish tinge seen in infectious rhinitis. Nasal membranes looked similar to those following the topical application of a vasoconstrictor agent such as neosynephrine. A second consistent feature was the patency of the nasal airway. The deeper structures of the nasal cavity were easily seen by anterior rhinoscopy without the use of vasoconstrictor agents. The openings of the glands of the nasal mucous membranes were not prominent. Membranes were not edematous and there was little vascular congestion or turgor. Purulent secretions, crusting or bleeding were not commonly observed. Two patients (2/87) had unilateral nasal polyps.

There were no distinctive changes observed in the oral cavity, external auditory canal or tympanic membranes or in the neck. No significant adenopathy was present and indirect mirror laryngeal examination was within normal limits in all patients.

Roentgenographic examinations

Paranasal Sinuses: Roentgenographs of the paranasal sinuses including Waters, Caldwell, submento-vertex and lateral views were obtained in each patient. Roentgenographs were considered to be abnormal in 16 patients (18%). Of these, a rudimentary frontal sinus was the only abnormality present in 2. Of the remaining 14, 2 had evidence of acute sinusitis manifested by air fluid levels, one in the left antrum, the other in the right frontal sinus. Four exhibited thickening of the mucosa of the antrum of mild to moderate degree without evidence of an air-fluid level. One had an antral osteoma and 3 had mucocoeles, one in the left antrum, one in the right antrum and one, bilaterally. Four exhibited bilaterally small, sclerotic well-aerated antra. Although there was no antecedent history of chronic sinusitus or treatment of sinus disease the radiographic interpretation of these changes in these latter four patients was that of chronic maxillary osteitis. One of the patients with nasal polyps had an air-fluid level in the frontal sinus. The other patient with nasal polyps had no abnormalities of the paranasal sinuses on roentgenographic examination. Of these 16 patients, only three had local unilateral disease of the sinuses which in time of onset and degree of severity could be related to the PIHH. These patients were included in this study because their local disease was unilateral.

Skull: Roentgenographs of the skull including frontal and lateral views were obtained in each patient. Two patients exhibited calcification of the falx cerebri while one exhibited hyperostosis frontalis interna. Roentgenographs of all other patients were considered to be within normal limits. No evidence of an olfactory cleft tumor was found in any patient.

Radionuclide studies: Brain scanning following an intervenous injection of $99^m$ technetium was carried out in each patient; while no intrinsic pathology of the brain was observed in any patient accumulation of $99^m$ technetium in the nasal region was observed on both frontal and lateral views in 40% of the patients. This accumulation was previously observed in 78% of a group of patien with Sjögren's syndrome, has been termed Rudolph Sign and has been previously been related to the intensity of inflammation in the nasal mucous membrane.

Taste: Median detection and recognition thresholds for the patients with PIHH were elevated above normal levels for salt, sour and bitter whereas for sweet, median detection and recognition thresholds were at the upper limit of normal. Forced scaling patterns for the patients were significantly different from those of the controls for each taste quality tested.

Smell: Median detection and recognition thresholds for the patients with PIHH for representatives of each of three vapors (pyridine, nitrobenzene and thiophene) were elevated above normal levels for each vapor tested.

Hearing: Air and bone conduction thresholds were obtained in 79 of the 87 patients on at least one occasion. Twenty-six of 79 patients (31%) had no history of hearing loss and had normal hearing thresholds upon testing. Thirty-five of the 79 (44%) gave a history of hearing loss prior to the onset of their influenza and had abnormalities demonstrated by testing. Eighteen of the 79 (25%) noted the onset of hearing abnormalities at or during the time of the influenza and hearing losses of various types were observed.

None of the 18 patients with hearing losses related in time to the influenza gave a history of otitis media at the time of the acute illness. Each of these 18 patients developed a sensorineural hearing loss, 8 with unilateral losses, 10 with bilateral losses. Ten (56%) exhibited a mild high frequency loss (loss of 30 dB or less in the speech range with further losses in the frequencies above 2000 Hz), 2 (11%) exhibited a moderate-severe high frequency loss (loss of 30-60 dB in the speech range), 4 (22%) exhibited a "notch" at 4000 Hz and 2 (11%) exhibited a "peaked curve".

Microscopic examinations

Taste buds: Taste buds from patients with PIHH were examined by light and electron microscopy and compared with taste buds removed from normal volunteers and from patients with various acute and chronic diseases in whom taste acuity was normal. Taste buds from patients with PIHH showed severe disruption of the pore region of the bud with marked diminution in the number and organization of the neurosecretory granules, absence of the dense, extracellular material, amputation of the finger-like projections from cells which extend into the pore region and the presence of many large and small vacuoles.

Nasal mucous membranes: Biopsies from the nasal mucous membranes of patients with PIHH were examined by light microscopy and compared with similar tissue taken from normal volunteers undergoing local nasal procedures for cosmetic reasons and from patients with various acute and chronic diseases in whom olfactory acuity was normal.

Biopsies from patients demonstrated thickening of the surface epithelium with mild cellular atypia, moderate to marked thickening of the basement membrane and, most characteristically, infiltration of the upper lamina propria with chronic inflammatory cells. These cells are mainly lymphocytes but plasma cells were also observed. Relatively little, if any, inflammation was present in the submucosal glands. Significant sclerosis and fibrosis of the upper lamina propria was also commonly observed. Both serous and mucous glands were decreased in number but serous glands were decreased to a much greater degree.

The diagnosis of PIHH may be made by an appropriate history, observing clinical findings in the nose, finding abnormalities in taste and smell perception, demonstrating specific changes in nasal mucous membrane biopsies and by demonstrating the presence of Rudolph Sign following intravenous injection of $99^{m}$ technetium. Together these findings are relatively specific for this disorder which is surprisingly common.

5. Treatment of patients with taste and smell dysfunction. A randomized, double blind crossover study of the effects of zinc sulfate and placebo was carried out in 106 patients with taste and smell dysfunction secondary to a variety of etiological factors. In the patient group prior to treatment mean serum zinc concentration and leukocyte alkaline phosphatase activity were significantly lower than normal. Results indicate that placebo was effectively equivalent to zinc sulfate in the treatment of these disorders although there was no change in mean serum zinc concentration or urinary zinc excretion among the placebo responders. Although these results demonstrate abnormalities of zinc metabolism in patients with taste and smell dysfunction they fail to provide evidence for a systematic, therapeutic approach to the many disorders underlying taste and smell dysfunction. However, the methods and procedures developed in this study demonstrate that taste and smell dysfunction can be studied in a quantitative, systematic manner.

Significance:

1. Anatomy. Details of the anatomical characteristics and functional significance of Type I, II and III cells in taste buds from fungiform and circumvallate papillae have been confirmed. Type III cells appear to be the receptor cell of the taste buds as determined by the direction of its synaptic connections, the Type II cell appears to function in a manner which protects the taste bud through its contractile mechanism and the Type I cell may supply the neurotransmitter material necessary for the initial neural events of the taste process to occur. The presence of acetylcholinesterase in the pore region of the taste bud confirms earlier hypotheses relating to the significance of the cholinergic nervous system in the taste bud.

2. Chemistry. A simpler process to purify miraculin has been developed which results in the yield of a much more purified glycoprotein. The properties of this glycoprotein havebeen studied. The sugar moeities of this glycoprotein have been confirmed as fucose, xylose, mannose and galactose and their ratios confirmed by thin layer and gas-liquid chromatography. The utilization of this glycoprotein in the control of sugar intake in man has been investigated and found to be particularly useful in the intake of sweetened beverages where it can be adapted to supply all the sweetness required for normal hedonic purposes.

The functional significance of taste bud membranes has been confirmed through the demonstration of specific binding and displacement of several sugars to these membranes. Each $^{14}C$ sugar studied behaved as did unlabelled sugar with respect to binding activity; specific binding of $^{14}C$ sugars to the taste bud membranes occurred but did not occur with the non-taste bud bearing epithelial membranes where only non-specific binding occurs. Inhibitors which bind metals or attack sialic acid residues significantly interfere with this binding indicating the importance of these two moeities in the binding process.

The role of nerve growth factor (NGF) in the taste process has been investigated. Specific displacement of this substance in isolated taste bud membranes has been achieved suggesting that a role for NGF can be identified at the taste receptor. NGF activity has been isolated in human parotid saliva as measured by bioassay, in chick sympathetic ganglia, and by radioreceptor assay, using dorsal root ganglia. This activity has been related to a purified fraction of human parotid saliva and is the first demonstration of a NGF type of activity in man not identifiable with tumor activity.

A zinc containing protein present in human parotid saliva was predicted in the annual report 1972-73. This protein has been identified, isolated, purified and some of its chemical characteristics identified. It has a molecular weight of 37,000 and contains 8% histidine per mole. The protein has been labelled in vivo with $Zn^{65}$ and constant specific activity with native Zn has been achieved. This protein has some properties in common with mouse NGF and has been shown to displace $I^{125}$ mouse NGF in binding studies with bovine taste bud receptor membranes. This protein is often significantly reduced in the saliva of patients with hypogeusia. Therapy with zinc ion may induce the formation of this protein and result in the alleviation of the observed symptoms of taste loss and taste dysfunction. This protein has been named gustin.

A total protein fractionation of human parotid saliva has been accomplished for the first time. This fluid has been fractionated into 6 major fractions with gustin a major component of Fraction II. A glycoprotein with several unusual molecular characteristics has also been identified in Fraction II. This protein is made up primarily of proline (1/3) and lysine (1/3) and may represent a form of procollagen. Studies in which gustin and the glycoprotein were combined have shown an enhancement of NGF activity suggesting that these proteins may exhibit some functional significance in combination which neither has individually. Protein markers have also been used to characterize each of the other fractions obtained from human parotid saliva. NGF activity is not present in these other fractions.



GIK

3. Physiology. The relationship between salt intake, estrogen and progesterone administration and hypertension was systematically evaluated. In the male rat although estrogen or estrogen plus progesterone administration produces an increase in salt intake it does not appear to be associated with the production of hypertension. On the other hand in preliminary studies administration of estrogen or estrogen plus progesterone to female rats is associated with both the increase in salt intake and the production of hypertension. These studies suggest that some differences in endocrine function may be important in understanding the mechanism by which these hormones produce these different effects in male and female rats. These results could lead to information of importance in understanding the mechanism by which various drugs used to control fertility influence blood pressure.

Studies in man clearly demonstrate that patients with essential hypertension exhibit a significantly greater preference for NaCl than do normotensive subjects. This preference is not related to taste, per se. Following effective treatment with antihypertensive agents preliminary studies suggest that salt preference in these patients decreases. Whether these changes are causally related are not presently known but these studies offer a useful, novel manner in which to obtain important information of usefulness to our knowledge of the physiology of salt intake and to its possible relationship to blood pressure.

4. Pathophysiology. An approach by which patients with disorders of taste and smell can be systematically investigated in order to evaluate the etiological factors responsible for their disorders has been established. It is now possible to provide a rational biochemical basis upon which to establish a differential diagnosis for these disorders. These novel techniques have been applied to several systemic disorders and offer aid in the differential diagnosis of these disorders (e.g., Sjögren's syndrome, allergic rhinitis, Wegener's granulomatosis and midline granuloma of the face). The diminution of the protein gustin in some patients with disorders of taste and smell has proven to be a useful clinical tool in these investigations and has allowed the diagnosis of central lesions affecting taste acuity to be distinguished from that of more local lesions which affect taste. The importance of metabolic factors in the production of abnormalities of taste following head trauma has been suggested for the first time and this hypothesis is substantiated by the finding of depressed levels of gustin in patients of this type.

Proposed Course of Project:

1. The identification of an acetylcholine receptor at the taste bud receptor membrane will be sought, in part through the utilization of the binding of α-bungarotoxin to the taste bud receptor membrane. Other biochemical techniques will be utilized to investigate the role of neurotransmitters at the taste bud membrane in vivo, in man and animals, and in vitro.

2. The systematic investigation of the relationships between salt intake, hormones and hypertension will be continued in studies to be performed in man and in animals.

3. The molecular characteristics of gustin will be investigated further. Its relationship with the salivary glycoprotein will be systematically studied.

4. A radioimmunoassay and a radioreceptor assay for gustin and for the salivary glycoprotein will be obtained. In this manner a definitive clinical test for the quantitation of these proteins and their relationship to taste acuity will be established.

5. A double-blind study will be undertaken (once the identification of an appropriate patient population is established through the utilization of the radioimmunoassay and radioreceptor assay) to establish the efficacy of zinc ion in the treatment of an appropriate population of patients with taste disorders.

6. The characteristics of human nerve growth factor will be established in relationship to the characteristics of gustin and the salivary glycoprotein. These characteristics will be compared to that of other growth factors including insulin, NSILA and epidermal growth factor.

Keyword Descriptors: Taste, Smell, Saliva, Parotid Proteins, Gustin

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1. Hypertension-Endocrine Branch
2. Section on Neuroendocrinology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Trace Metal Metabolism

Previous Serial Number: NHLI 105(c)

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Cooperating Units: National Cancer Institute, Nuclear Medicine Branch, Clinical Center, NINDS, NIMH, Metabolic Disease Branch, NIAMDD, Baylor University, School of Medicine, Houston, Texas, St. Christopher's Hospital for Children, Philadelphia, Pa.

Project Description:

Objectives: To study the physiology, metabolism, biochemistry and pathology of copper, zinc and other trace metals in physiological fluids and tissues of normal subjects, in patients with various diseases and in animals. These studies include the interaction between metals and their binding proteins.

Major Findings:

Biochemistry: Estimation of zinc concentration in parotid saliva by flameless atomic absorption spectrophotometry in normal subjects and in patients with idiopathic hypogeusia. A relationship between taste acuity and zinc metabolism has been previously established in that zinc depleted patients exhibit hypogeusia (loss of taste acuity); treatment with zinc ion returns this hypogeusia toward or to normal in some of these patients. These changes in zinc metabolism were reflected initially, in some patients in the untreated state, in lower than normal levels of zinc measured in serum and hair and with a return to normal following treatment with zinc ion. Although these general relationships were clearly established in some patients there were many untreated patients with hypogeusia in whom serum zinc concentrations were within normal limits yet whose hypogeusia was amenable to treatment with zinc ion. These results suggested that serum and hair zinc reflected changes in taste acuity and zinc metabolism in only a very general manner, primarily in cases of severe zinc depletion.

A close relationship between taste acuity and the presence of saliva has been previously established in that patients with xerostomia of several etiologies exhibit hypogeusia; treatment of these underlying conditions with several agents which returned salivary flow toward or to normal was associated with the return of taste acuity toward or to normal.

Since saliva and zinc both appeared to be important factors in the control of taste acuity, it seemed useful to measure the zinc concentration of saliva in subjects with normal taste acuity and in patients with hypogeusia in an attempt to obtain a closer correlation, if it did exist, between zinc levels and taste acuity.

Estimation of salivary zinc concentration by conventional flame aspiration atomic absorption spectrophotometry (AAS) was attempted first but it proved impractical since salivary zinc concentrations were often below or near the sensitivity of the instrument and a rather large amount of sample was required since it was necessary to measure the sample without dilution. To overcome these problems we measured salivary zinc concentration by flameless AAS. ' A method by which salivary zinc concentrations were estimated by flameless AAS was developed and applied to subjects with normal taste acuity and in patients with hypogeusia. By this technique zinc was determined rapidly and reproducibly in concentrations below 1 ppb (1 ng/ml) in a sample as small as 5 µl. By this technique significantly less zinc was measured in the saliva of patients with hypogeusia compared with that in subjects with normal taste acuity ($p < 0.001$, Table I).

TABLE I

| Group | No of Subjects | Age Range | Zn ppb |
|---|---|---|---|
| Normal Volunteers | 34 | 16-65 | 51 ± 14+ |
| Men | 11 | 23-64 | 53 ± 11 |
| Women | 23 | 16-65 | 50 ± 16 |
| All Caucasians | 29 | 16-65 | 49 ± 14 |
| All Blacks | 4 | 34-55 | 58 ± 14 |
| Patients with hypogeusia | 47 | 19-73 | 10 ± 6* |

+Mean ± 1 SD

*Significantly below mean of normal volunteers ($p < 0.001$)

Physiology

1. Role of zinc in taste, food intake and growth. Preference for HCl, food intake, growth and changes in zinc metabolism were studied in 4 groups of rats fed either zinc deficient or zinc replete diets ad libitum or by force. Group A was fed, ad libitum, a zinc deficient diet; group B was pair fed with group A a zinc supplemented diet; group C was fed, ad libitum, a zinc supplemented diet; group D was force-fed a zinc deficient diet in an amount similar to that taken by group C. Mean plasma zinc concentrations and 24 hour urinary zinc excretion was measured in each group. At the termination of the experiment organ weights of each group were also measured.

Plasma zinc and urinary zinc excretion were significantly lower in groups A and D (receiving zinc deficient diets) than in the controls (group B and C). HCl preference for each group (A and D) given zinc deficient diet was significantly greater; i.e., they demonstrated decreased aversion, than did the controls suggesting decreased taste acuity in the zinc deficient groups. Growth rates of the rats of group A was significantly below that of the rats fed zinc supplemented diets (groups B and D) but also below that of rats force-fed zinc deficient diet. Testicular weight was significantly lower than in controls in rats fed zinc deficient diet ad libitum or by force. These observations indicate that some sequellae of zinc deficiency relate to the metal deficiency, per se, (i.e., taste changes, anorexia) whereas others relate more closely to changes in food intake (body weight, testicular changes).

2. Cerebellar dysfunction, mental changes, anorexia, and taste and smell dysfunction associated with acute zinc loss: a new syndrome. L-histidine was administered orally in graded doses to 4 women and 2 men with progressive systemic sclerosis. During the administration of this amino acid a constellation of clinical changes occurred following a decrease in serum zinc concentration and an increase in urinary zinc excretion. These changes were the production of anorexia, taste and smell dysfunction, organic mental and psychiatric symptoms including depression and frank psychosis and cerebellar dysfunction including intention tremor, positive Romberg sign and ataxia. Following discontinuation of the amino acid no rapid changes of these symptoms occurred. Following administration of zinc ion, with or without the continued administration of the amino acid L-histidine, all symptoms and signs and metabolic changes were consistently reversed within 24-48 hours. These studies suggest that zinc plays an important role, not only in appetite and taste and smell function but also in cerebellar function as well. These studies have also been now carried out in patients with chronic zinc depletion with similar results.

3. Zinc$^{65}$ metabolism in man. Zinc absorption, distribution and excretion was studied in 11 patients with hypogeusia of various etiologies, parents of a patient with Wilson's disease and a patient with cystinuria. Zinc$^{65}$ was administered orally (1-2μCi) or intravenously (IV)(15-24 μCi) while patients were on a constant diet. Patients with hypogeusia were given $Zn^{++}$ (100 mg/day) or placebo during the study. Zinc$^{65}$ activity in blood, urine, liver and thigh was determined and total body retention was measured for 207 to 800 days after administration. Following IV injection, the concentration of zinc$^{65}$ in whole-

blood and plasma decreased rapidly (<2% in plasma, <5% in whole-blood after 24 hours). Red blood cell concentration reached maximum 7-10 days after injection, then gradually decreased. External gamma-ray measurements over the liver area showed maximum value (50-75% of injected zinc$^{65}$) between 5 and 24 hours, then gradually decreased. Activity measured over the thigh area decreased initially, then increased to maximum values during the next 20-40 days. Following oral administration of zinc$^{65}$, three absorption patterns were observed: 6 patients absorbed 65 ± 4% (M ± SEM); 3 patients absorbed significantly less (28 ± 9%, $p < 0.01$) and 2 patients absorbed significantly more (99 ± 1%, $p < 0.001$). Total body retention fit a two component exponential pattern with biological half-time of 8 and 300 days. The longer half-time component was shortened in 1 patient with congenital hypogeusia and 2 patients while receiving exogenous zinc. This effect was not seen in other patients given exogenous zinc.

These results may be applied directly to an understanding of zinc metabolism in several disease states. Lombeck et al., reported the reduction of oral absorption of $Zn^{65}$ in patients with acrodermatitis enteropathica and suggested this abnormality as the cause of the signs and symptoms of observed zinc deficiency in this condition. In the two untreated patients they studied absorption of $Zn^{65}$ was indeed low (patients; 15%, 30%: normals; mean, 66%, range, 58%-77%). They also stated that the elimination rate of absorbed $Zn^{65}$ was similar in both patients and controls clearly delineating an absorptive not a retentive defect of zinc. However, in the one treated patient they studied $Zn^{65}$ absorption was much higher, 42%. Thus, following treatment, patients with acrodermatitis enteropathic may exhibit an increased oral absorption of $Zn^{65}$ in contrast to the usually observed decreased $Zn^{65}$ absorption observed in normal subjects given exogenous zinc.

Results of our total body absorption indicated three distinct absorptive patterns as noted above. One group (A), absorbed 99 ± 1% (Mean ± 1 SEM) of the administered dose; one group (B), absorbed 65 ± 4% of the dose, range 51-77%; a third group (C), absorbed 28 ± 9% of the dose, range 8-37%).

Group B, which comprised the largest number of patients, exhibited absorption in a range quite similar to that noted by Lombeck et al. in their small group of normal subjects. However, absorption in Group C was quite similar to that noted in their patients with untreated acrodermatitis enteropathica yet our patients exhibited, as their only functional complaint, disorders of taste and smell; although the mean level of serum zinc in our patients was significantly lower than normal as was the alkaline phosphatase activity of their blood leukocytes none had any of the clinical manifestations of acrodermatitis enteropathica.

Evaluation of these results suggest that impaired zinc absorption is not the primary defect in acrodermatitis enteropathica. Data from Lombeck et al. and from our group would suggest that the defect in acrodermatitis enteropathica lies in the genetic lack of an appropriate gastrointestinal transport moeity (polypeptide or protein), a lack which is somehow corrected through the administration of oral zinc, even in very small quantities which may be only

slightly higher than the presently accepted Recommended Daily Allowances. This would suggest that zinc acts in this disease to either induce the formation of this transport moeity, to inhibit its repression, etc. If this hypothesis were correct then the pathological sequellae of acrodermatitis enteropathic may be explained in full. Absorption in the presence of small amounts of dietary zinc would be low producing the sequellae of zinc deficiency in blood and tissues; absorption in the presence of larger amounts of zinc would increase gradually, correcting these abnormalities since the remainder of the binding and transport system on the blood side of the gut wall appears to be intact. This hypothesis would also explain the sometimes observed lag phase in the production of symptoms with the stopping of oral zinc supplementation.

The role of impaired zinc absorption in patients with hypogeusia and hyposmia is more complex. In these patients there appears to be at least two defects in zinc metabolism, the one involving an impairment absorption across the gut wall in some patients and another involving an impairment in the formation of the zinc containing parotid protein gustin, a protein which has been suggested to act by stimulating growth and development of taste buds.

Continuation of research into these important areas have been hindered by several difficulties such as the practical measurement of zinc in several body tissues, the difficulty of serum or urine zinc levels to reflect accurately body zinc levels, sparsity of total body counting equipment with which to carry out dynamic studies of zinc metabolism and vagaries in the measurement techniques themselves. Lombeck et al., reported no measurable $Zn^{65}$ excreted in the urine in their patients with acrodermatitis enteropathica over the first 4 days of their studies. These results may be simply due to the technical failure to measure radioactivity in the entire 24 hour urine sample collected since in our patients with hypogeusia and hyposmia, even among those who absorbed less than 10% of the administered dose, measurable amounts of $Zn^{65}$ were excreted within the first 24 hours. Indeed, measurement of urinary zinc excretion over the first 24 hours of our studies after a small oral load of $Zn^{65}$ appears to supply an useful approximation of zinc absorption. Comparison of the total body absorption of $Zn^{65}$ calculated on the basis of balance data with the simple urinary excretion of $Zn^{65}$ during the first 24 hours after administration indicates the correlation between these two techniques is highly significant ($r = +0.810$, $p < 0.001$). Calculations of zinc absorption on the basis of balance data compared with that using only the data obtained from the urinary excretion of $Zn^{65}$ over the first 24 hours agrees very closely over a range of absorption from 8%-100%; signed differences between absorption calculated by these two methods are less than 1% whereas on an absolute basis differences are approximately 15%.

4. Role of parathyroid hormone in copper metabolism. Copper metabolism was studied in 8 patients with well documented hyperparathyroidism before and after removal of their parathyroid adenomas. In each patient prior to surgery serum copper levels were significantly elevated above normal (patients, 155 ± 6 µg/dl, M ± 1 SEM; controls, 106 ± 2) whereas urinary copper excretion was approximately 3-10 times normal (patients, range 50-400 µg/24 hours; controls, 20-70 µg/24 hours). Within three-five days following surgical removal of the

parathyroid adenoma urinary copper excretion returned to normal levels. This return was independent of urinary changes in adenyl cyclase, calcium, phosphorus, sodium or potassium. These studies suggest that parathyroid hormone may play some specific role in the control of urinary copper excretion and thereby in the control of copper metabolism.

5. Copper and zinc metabolism in patients with breast carcinoma. Serum copper and zinc concentrations were measured in seventy patients with breast carcinoma at varying stages of the disease and under varying conditions of treatment. Mean serum copper concentrations were significantly elevated above normal (patients, 162 ± 10 µg/dl, mean ± 1 SEM; controls, 106 ± 2) whereas serum zinc concentration was significantly lower than normal (patients, 72 ± 5 µg/dl; controls, 96 ± 2). Many of these patients exhibited anorexia and taste and smell dysfunction. Whether elevated serum copper and lowered serum zinc concentrations may serve as a diagnostic or therapeutic aid in the understanding of this disease is at present under investigation.

6. Copper and zinc metabolism in hypothyroidism. Serum, urine and salivary concentrations of zinc and copper have been measured in 5 patients with hypothyroidism before and after therapy with thyroid hormone. Serum and urine concentrations of zinc and copper have also been measured in 15 rats made hypothyroid with $I^{131}$ and in 15 control rats. In man serum zinc and saliva concentrations were below normal off treatment and returned toward normal after therapy with thyroid hormone. In rats serum zinc concentration was significantly lower than normal whereas urinary zinc excretion, expressed per mg of creatinine, was significantly higher than normal.

7. Copper and zinc metabolism in unmedicated acute and chronic schizophrenia. Patients with schizophrenia have been reported to exhibit several abnormalities of zinc and copper metabolism and these changes have been related to various therapies to aid in the treatment of these disorders. Each of these studies suffers from severe procedural limitations and the conclusions were not justified on the basis of the data obtained. In order to obtain a systematic evaluation of this important problem copper and zinc levels were studied in 30 patients with unmedicated acute and chronic schizophrenia in various tissues and related to their disease states where possible.

Copper and zinc levels were measured in 5 tissues as noted in Table II.

TABLE II

| | | SERUM | | | URINE | | CSF | | GASTRIC FLUID | | HAIR |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Condition | Number | Zn µg/dl | Cu µg/dl | Ceruloplasmin ng/dl | Zn µg/24° | Cu | Zn µg/dl | Cu | Zn µg/dl | Cu | Zn µg/dg |
| Patients | 20 | 92 ± 3 | 93 ± 5 | 34± 1 | 286±35 | 30±3 | 3±1 | 4±1 | 42±4 | 7±2 | 136±6 |
| Controls | 85 | 96 ± 2 | 106 ± 2 | 30± 2 | 353±23 | 36±5 | 7±2 | 8±2 | 40±5 | 10±3 | 180±4 |

The results of these studies indicated that patients with schizophrenia do not exhibit any change from normal in copper or zinc levels in serum, urine, CSF or gastric fluid. However, levels of zinc in hair were uniformly below the normal mean and the mean level was significantly lower than normal ($p < 0.001$).

In order to evaluate these findings further a second study was begun in which 10 additional patients were studied and also in which a group of patients with schizophrenia treated with various tranquilizing agents were studied. This work was undertaken since it is possible that long term therapy with some tranquilizing agents may itself produce zinc loss. This problem arises since any drug with an imidazole group may act as a zinc chelator and produce a long term loss of total body zinc as measured by hair zinc. These studies are in their final stages.

8. The Environmental Protection Agency (EPA) Panel on Zinc. The report on zinc for the EPA has been completed and the draft returned from its first reviews. The first draft of this work was distributed to the parent panel within the National Academy of Science in March. Initial results indicate that fish are significantly sensitive to low levels of zinc which may appear in the water secondary to the improper handling of untreated or even treated human and animal waste products; this effect produces a respiratory death and may be one cause of the rapid depletion of fish in areas where they were previously plentiful. In man, in children a new syndrome of zinc toxicity has been described consisting of lethargy, fatigue and anemia. Treatment with chelating agents has been useful in the reversal of these symptoms. The role of zinc in growth and development in man has been outlined.

9. Investigation of the copper defect in Menkes Kinky Hair Disease (MKHD). MKHD is a genetic, X-linked disorder, characterized by failure to thrive, seizures, progessive cerebral deterioration, pili torti, degeneration of blood vessel walls, deficiency of tissue copper and decreased activity of several copper dependent enzymes. Serum albumin and renal function are normal. Three patients with MKHD were investigated under four conditions: (1) untreated, (2) treated with 1.5 mg $CuSO_4$ intravenously, (3) treated with 10 mg $CuSO_4$ orally for 10 days and (4) treated with 10 mg copper nitrilotriacetic acid (CuNTA) orally for 10 days. Without treatment serum copper and ceruloplasmin, respectively, were less than 1/4 normal (patients, M ± 1 SEM, 24 ± 1 µg/dl, 4 ± 1 mg/dl; normal, 110 ± 2 µg/dl, 34 ± 2 mg/dl), while urinary copper excretion was more than four times normal (patients, 34 ± 5 µg/24°; normal, 7 ± 1 µg/24°). Intravenous $CuSO_4$ rapidly increased serum copper and ceruloplasmin slightly, as previously reported, transiently increased further the already increased urinary copper excretion but produced little if any amelioration of the existing tissue copper deficiency. Oral $CuSO_4$ did not alter significantly either serum or urinary copper or ceruloplasmin because it is mainly absorbed into the gastrointestinal mucosa, rather than across the gastrointestinal tract. Oral CuNTA increased serum copper and ceruloplasmin. Since copper is transported into cells by first order kinetics only after forming a relatively small molecular weight organometallic complex, copper in ceruloplasmin, due to its high association constant, is unavailable

for this transport whereas CuNTA is. Conceivably CuNTA could fulfill the necessary requirements for tissue.

Significance:

1. Transition metal ion absorption in the gastrointestinal tract. Based upon studies in normal man with $Zn^{65}$ and in patients with Menkes-Kinky Hair Disease an unified theory of the manner by which transition metal ions are absorbed across the gut has been proposed. This involves the absorption of a small molecular weight organometallic complex across the gut mucosa in a manner which follows first order kinetics, is then passively absorbed into the blood and transported by mechanisms previously shown.

2. Role of zinc in growth and development. The immediate major role of zinc in growth is through its control of food intake. This hypothesis was suggested previously from our earlier work and has been confirmed by studies in which rats were force fed zinc deficient diet and results compared with rats fed zinc deficient diet ad libitum. Results demonstrate that serum zinc concentrations in rats fed zinc deficient diet ad libitum or by force were similar and significantly below those of rats fed zinc supplemented diets. Similarly both groups of rats fed zinc deficient diet exhibited hypogeusia. However, the force fed rats grew at a significantly greater rate than did the rats fed zinc-deficient diet ad libitum indicating the role of anorexia in this process. In addition testicular weights of the force-fed rats were significantly greater than those of the ad libitum fed rats indicating that food intake rather than zinc deficiency per se is an important factor in this finding. These studies are the first to separate the specific roles of zinc affecting food intake and tissue changes.

3. Role of copper in Menkes-Kinky Hair Disease. This disease is at present difficult to treat since the mechanisms underlying this disease are poorly understood. We have proposed an hypothesis for the etiology of this disease, including the observed blood vessel changes, based upon biochemical considerations; i.e., these patients lack the small molecular weight organometallic complex which is necessary to produce normal copper utilization by tissues. To verify this hypothesis copper was conjugated with nitrilotriacetic acid and this complex was given orally to 2 patients with this disease. In each case there was an increase in serum copper concentration suggesting that copper crossed the gut wall. These studies identify a possible biochemical defect which could be responsible for some of the symptoms of the disease and are the first to identify a possible oral mode of treatment for this disorder.

4. Measurement of zinc in saliva and its meaning in health and disease. The development of this technique represents the first successful attempt to measure zinc accurately in human saliva. The technique is simple, rapid, inexpensive, accurate and reproducible. This tool allows the screening of large numbers of patients with various oral and metabolic abnormalities. It also allows for a rational differential diagnosis of hypogeusia to be made in that patients with normal salivary zinc levels may not exhibit hypogeusia on the

basis of salivary factors which influence taste buds. With this technique it is possible to specify local disease of the oral cavity or specific protein abnormalities of the saliva factors in the production of altered taste acuity.

5. Possible control of copper metabolism by parathyroid hormone. Studies in patients with hyperparathyroidism secondary to parathyroid adenomas suggest that parathyroid hormone per se influences urinary copper excretion. This effect may be either (1) through the mobilization of copper from its sites in bone and its passive excretion in the urine, (2) a specific action of parathyroid hormone on the renal tubule related to the tubular reabsorption of copper or (3) a specific action of parathyroid hormone on metallothionine or another, as yet unidentified, enzyme important in copper conservation in the kidney. Although the site of action is not yet understood these studies demonstrate for the first time a role for parathyroid hormone in copper metabolism and allow for a study of the specific locus of action of this hormone on copper metabolism.

6. Role of zinc in cerebellar and sensory function. The acute loss of zinc from the body is associated with the production of cerebellar changes including intention tremor and ataxia as well as changes in sensory function. These changes are all rapidly and completely reversed following the administration of zinc ion. Recent studies in Houston have indicated that hypogeusia, hyposmia and cerebellar dysfunction associated with zinc loss may appear on a chronic as well as on an acute basis and that some of these signs and symptoms may be reversed by zinc administration. Other studies indicate that zinc, which is the fourth most prevalent ion in the brain, may be readily removed and replaced from its sites in brain. These sites include hippocampus and cerebellum. The role of zinc in neural function has received much attention recently by Barbeau and others who have suggested that zinc metabolism may be important as a factor in epilepsy. Since the cerebellum appears to play an important role in the control of seizure activity the role of zinc in these tissues may lead to a new understanding of the interrelationships between cerebellar function, seizure activity and zinc metabolism.

7. Possible role of histidine and zinc metabolism in schizophrenia. Experimental production of schizophrenic like symptoms in man have been investigated by several workers in an attempt to understand the mechanisms of these puzzling diseases. Administration of the amino acid methionine to patients with schizophrenia exaccerbate the symptoms of these already effected patients. However, administration of the amino acid histidine produces symptoms which may be interpreted as those of an acute psychosis in previously mentally normal subjects; these symptoms are quickly and completely reversed by administration of zinc ion. These findings may lead to a new biochemical approach to the understanding of these diseases.

8. A simple test for measurement of zinc absorption in man. Urinary $Zn^{65}$ excretion after a small oral dose of $Zn^{65}$ is directly proportional to the absorption of the metal over a range of absorption from 8%-100%. This technique may make the evaluation of zinc absorption in man a practical and simple test by which the present vagaries of zinc metabolism may be systematically evaluated.

Proposed Course of Project:

1. The kinetics studies of $Zn^{65}$ and $Zn^{69}$ in man will be continued in order to quantitate in more detail the manner by which zinc is absorbed, distributed and excreted in man in health and in disease.

2. The specific interrelationships between copper and parathyroid hormone will be investigated and the locus of its action identified and characterized.

3. The treatment of patients with Menkes-Kinky Hair Disease with copper -NTA on a long term basis will be carried out through collaboration with Dr. W.D. Grover, Temple University, Philadelphia, Pa. Problems of therapy will be evaluated and mechanisms of action will be verified. Blood vessel changes will be studied in particular detail.

4. The book Zinc written under the auspices of the National Academy of Sciences and the Environmental Protection Agency will be completed. It will represent the state of the art of our present knowledge of zinc at all levels of biological importance. It will be published by the National Academy of Sciences in 1975.

5. Salivary zinc levels will be measured in patients with various diseases and correlated with taste acuity in order to evaluate the correlation between these two measurements and to evaluate the use of salivary zinc levels as a useful index in the differential diagnosis of hypogeusia.

6. Further studies in the role of zinc in cerebellar function will be carried out at the clinical level. The ability of zinc to cross the blood brain barrier and bind to specific brain sites will be investigated in an effort to understand the role this ion plays in brain function.

7. A systematic investigation of the potential role of histidine and zinc in schizophrenia will be evaluated in a double blind study to be undertaken within the NIMH this fall.

Keyword Descriptors: Copper, Zinc, Zinc metabolism, Zinc absorption, Menkes-Kinky Hair Disease.

Honors and Awards: None

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Project No. Z01 HL 01983-12 HE
1. Hypertension-Endocrine Branch
2. Section on Neuroendocrinology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Hormonal Effects on Sensory and Neural Function

Previous Serial Number: NHLI-104(c)

Principal Investigator: Robert I. Henkin, M.D., Ph.D.

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Cooperating Units: National Institute of Mental Health, University of Connecticut, School of Dental Medicine, Farmington, Connecticut, College of Physicians & Surgeons, N.Y., N.Y.

Project Description:

Objectives: To investigate systematically the interrelationships between steroid hormones, thyroid hormones, electrolytes and psychodelic agents on neural and sensory function with respect to the manner by which sensory signals are detected and integrated by sensory receptors, neuraxons and brain. In addition, to investigate the relationship between steroid and protein hormones and amino acids and sleep patterns.

Major Findings: 1. Central and peripheral nervous system changes related to changes in endogenous secretion of adrenal corticosteroids in man.
Averaged evoked cortical responses (AER) using a graded intensity series of visual and auditory stimuli were obtained in 9 patients with adrenal cortical insufficiency and in 3 with panhypopituitarism while each was maintained on adequate replacement therapy, while each was off all adrenocorticosteroid replacement therapy for 4-9 days and while each was replaced with deoxycorticosterone acetate, 20 mg daily for 2-4 days. Patients with adrenal cortical insufficiency were also studied off all replacement therapy while receiving intravenous infusions of ACTH (40 units) in 5% dextrose and water or 5% dextrose and water without ACTH over an 8 hour period. Under the same conditions measurements of eye movements, light touch, manual and oral stereognosis, and rod and frame manipulation were obtained. Results indicated that, as a group, while untreated, AER were greater in amplitude than while treated with adequate replacement adrenocorticosteroids. Treatment with deoxycorticosterone (DOCA) acetate did not alter these changes in any manner. Administration of ACTH during the period off all steroid replacement was accompanied by a decrease in amplitude of the AER. Treatment with replacement carbohydrate-active steroid alone returned these changes to normal within 24-48 hours.

While off all adrenocorticosteroid therapy these patients also showed inability to perform oral stereognosis tasks, manipulate rod within a frame to obtain a perpendicular placement yet were extremely sensitive to the placement of nylon filaments upon their skin, recognizing skin deformation at a significantly lower pressure than that of normal volunteers. Treatment with DOCA did not alter these findings in any manner but replacement therapy with carbohydrate-active steroid returned these changes to or toward normal in each patient.

2. The effects of ACTH on human sleep patterns. The effects of ACTH on human sleep was studied in 9 healthy normal volunteers by administration of ACTH (40 units intravenously) beginning either at 8 a.m., 3 p.m. or 11:30 p.m. and continuing over a period of 8 hours. ACTH was also administered to 3 patients with treated and untreated adrenal cortical insufficiency in the same manner over 8 hours beginning at 8 a.m. ACTH produced a significantly greater reduction in REM sleep in the normal volunteers than in the patients with Addison's disease, as expected. During the overnight infusion beginning at 11:30 p.m., approximately 4 hours of continuous infusion of ACTH was required before REM sleep was reduced. The REM suppressive effect of ACTH appeared to attenuate about 12 hours following the termination of the ACTH infusion. In addition, ACTH significantly reduced total sleep time in the volunteers following infusions beginning at 8 a.m. and 3 p.m. but not when ACTH was infused during sleep. Delta sleep was also reduced following the 8 a.m. infusion in the volunteers.

These results suggest that ACTH affected sleep in the normal volunteers through its effect on adrenal corticosteroid secretion. Time appears to be an important aspect of the effect of ACTH and adrenocorticosteroids upon the central nervous system. The effects of ACTH on the sleep patterns of patients with Addison's disease, off treatment, suggests that the protein hormone may play some direct role in altering central nervous system function.

3. The effects of L-histidine on the sleep-waking cycle in man. L-histidine was administered to 3 patients with intractable narcolepsy at a dose of 20 gm/day for 2 weeks, to 4 normal volunteers at a dose of 32.4 gm/day for 5 days and to one patient with scleroderma at a dose of 48.6 gm/day for 16 days. No effect on nocturnal sleep patterns in any of the patients or subjects or on the symptoms of narcolepsy was observed. Because of the metabolism of histidine to histamine these results fail to support the hypothesis that histamine is a "waking factor".

4. The role of thyroid hormone in taste and smell sensation. In order to study the specific role of thyroid hormone in taste function fifteen adult male rats were made hypothyroid by treatment with $I^{131}$ (300 μCi/100 gm body weight) matched with 15 control rats, each caged individually and offered a choice between water and graded concentrations of sucrose, quinine, NaCl and HCl. Hypothyroid rats exhibited increased preference for NaCl ($p < 0.001$) and decreased aversion for quinine ($p < 0.001$) than did the untreated controls but exhibited an increased preference for sucrose ($p < 0.001$). There were no significant

differences between the controls and the hypothyroid rats for HCl. After parenteral administration of thyroxine to the hypothyroid rats, 10 µg/100 gm body weight for 2-3 weeks preference for NaCl, sucrose and quinine returned to normal.

Following treatment with exogenous thyroid hormone each of these taste changes reverted to normal. The changes occurred within 1 week of hormone administration and persisted as long as the hormone was administered. These studies indicate that hypothyroidism produced by $I^{131}$ in rats is associated with significant alterations in taste and may serve as a useful model for studies in man.

In patients with hypothyroidism taste and smell function had been previously evaluated. Hypogeusia, hyposmia, dysgeusia and dysosmia had been previously observed while off treatment reverting to or toward normal during replacement. In an effort to evaluate the pathological changes which might underlie these findings surgical removal of lingual circumvallate papillae was carried out in 3 patients with untreated hypothyroidism and examined by light and electron microscopy. Whereas as many as 100 taste buds may be observed in circumvallate papillae from subjects with normal taste acuity no taste buds have been observed in any of the papillae examined in the patients with untreated hypothyroidism.

Significance: Role of steroid, ACTH and thyroid hormones in neural function.

Steroid hormones: Carbohydrate active steroids affect neural function at each locus of the nervous system, at the receptor, the nerve and the central nervous system. These effects have also been observed in sleep.

ACTH: ACTH has an effect on central nervous system function apparently independent of its action on the secretion of adrenocorticosteroids. This has been observed in patients with adrenal cortical insufficiency, off treatment, who exhibited significant changes in their sleep patterns after receiving ACTH without any change in their secretion of adrenocorticosteroids. ACTH effects on sleep were not observed in normal volunteers in whom ACTH was administered during the sleep period itself but significant effects were recorded when ACTH was administered 8 hours prior to the sleep recordings. These results in normal subjects suggest that ACTH can effect sleep through its effects on adrenocorticosteroid secretion but that time is an important aspect of these effects.

Thyroid Hormones: Decreased thyroid hormone produces dysfunction in taste and smell acuity (hypogeusia, dysgeusia, hyposmia and dysosmia) as well as decreased auditory acuity, decreased axonal conduction velocity and increased synaptic delay. These results are similar in all respects to those observed in patients with Cushing's syndrome in whom excessive secretion of carbohydrate-active steroid has been measured. Replacement with thyroid hormone is associated with the return of each of these sensory and neural abnormalities toward or to normal. Studies on taste were also carried out in hypothyroid animals untreated and after treatment with replacement thyroid hormone. Results were similar to

those observed in man.

Sleep studies: ACTH and carbohydrate-active steroid play significant roles in the control of delta sleep as well as REM sleep.

Proposed Course of Project:

1. To define further the specific role of ACTH and other protein hormones on sensory and neural function.

2. To demonstrate the specific role of thyroid hormone on taste receptor and neural function.

Keyword Descriptors: Adrenocorticosteroids, Thyroid Hormone, ACTH, Sleep, Averaged Brain Evoked Responses, Taste, Smell.

Honors and Awards: None

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ANNUAL REPORT OF THE
MOLECULAR DISEASE BRANCH
NATIONAL HEART AND LUNG INSTITUTE
July 1, 1974 through June 30, 1975

The research program of the Molecular Disease Branch is as in the past directed primarily toward the elucidation of the chemistry and metabolism of the plasma lipoproteins with the objective of defining and ultimately treating the derangements of lipid transport and metabolism that lead to atherosclerosis and numerous other diseases in man. Some of the specific areas of progress in the past year are outlined below. In addition, we are continuing a collaborative investigation of the structural, biological and immunochemical properties of parathyroid hormones and related peptides.

Basic to any understanding of the structure and function of the plasma lipoproteins, normal and abnormal, is a knowledge of the chemistry of the smaller molecules that they contain. The protein components (apoproteins) which, as a group, serve to promote the transfer of lipid from cells and to stabilize it in the circulation obviously present the greatest challenge in this regard. The primary structure of the A apoproteins from human plasma have been established, A-II here and A-I elsewhere. The three C apoproteins were discovered here and the primary structure of C-I and C-III determined in past years. Work on the structure of C-II has now progressed to the point where sequencing and alignment of peptide fragments (generated enzymatically and chemically) is about 75% completed. It has been found that, unlike the intact C-II apoprotein, none of these peptides is capable of activating lipoprotein lipase.

The quaternary structure of the intact lipoproteins is determined by the nature of the interactions between their constituent apoproteins and lipids. We reported last year several important observations on the configuration and orientation of the A apoproteins in human high density lipoproteins (HDL). These studies, employing physical methods for measurement of molecular properties of proteins and lipids ($^{13}C$ and $^{31}P$ nuclear magnetic resonance, circular dichroism, fluorescence, difference absorption spectroscopy), have continued in collaboration with members of the Laboratory of Chemistry, NHLI, and the Clinical Endocrinology Branch, NIAMDD. From investigations of the characteristics of the individual A apoproteins in aqueous solution, after recombination in vitro with specific phospholipids and in their normal state in native HDL we have obtained considerable new evidence in support of the molecular structure for HDL that was suggested last year. In this model HDL is a spherical micelle (ca. 120 Å in diameter) consisting of lipids with the polar groups of the phospholipids at the surface (aqueous interface) and with the globular A apoproteins which exhibit a high degree of ordered (helical) structure, partially imbedded or "floating" in a "sea" of lipid. It appears that the major forces in the protein-lipid interactions are hydrophobic and that interactions between the A-I and A-II proteins could be of special importance in the organization of the native HDL molecule. Observations on the self-association of apoprotein A-II suggest that it may be an A-II dimer that is specifically involved in lipid binding.

We had found in the past that A-I in the absence of A-II was unable to bind the phospholipids lecithin and sphingomyelin to any significant extent. In recent studies, however, it has been shown that A-I avidly binds lysolecithin in a fashion seemingly related to the primary structure of this apoprotein. The functional significance of this association is unknown at present, but it is noteworthy that apoprotein A-I is reported to be an activator of the enzyme lecithin:cholesterol acyl transferase which catalyzes the transfer of a fatty acid from lecithin to cholesterol in a reaction that generates lysolecithin.

In continuing investigation of factors that influence synthesis and degradation of the human plasma lipoproteins through kinetic analyses of data obtained after administration of radioactively labeled apolipoproteins, we have benefitted greatly from a close collaboration with the Laboratory of Theoretical Biology, NCI. Last year it was shown that the B and C apoproteins of the very low density lipoproteins (VLDL) turn over independently and transfer separately through two distinct compartments of the circulating lipoproteins. In addition, it was found that in patients with type III hyperlipoproteinemia production of apoprotein B is accelerated, exceeding the capacity of the body to metabolize VLDL through the normal pathway and probably causing the accumulation of abnormal VLDL that is characteristic of this disorder. Current studies are designed to obtain data that will enable us to describe HDL metabolism, or at least the metabolism of its A-I and A-II apoproteins, in terms of the multicompartmental model of human lipoprotein metabolism that has been developed from the earlier work. Computation of rates of synthesis and fractional catabolic rates for the HDL apoproteins has indicated that apoproteins A-I and A-II turn over at the same rate. These studies are consistent with the view that the HDL macromolecule is metabolized as a whole without preferential removal of individual apoproteins. In addition, it has been shown that the fall in plasma HDL content that occurs in normal humans following ingestion of a diet high in carbohydrate is the result of increased catabolism while the rate of synthesis remains unchanged. The metabolism of HDL is of particular interest because A-I, a putative activator of lecithin: cholesterol acyl transferase, is the predominant protein component and it has been suggested that high density lipoproteins may play a critical role in the removal of cholesterol from tissues. The HDL further serves as a reservoir for the C apoproteins (one of which (C II) is the activator of lipoprotein lipase) between tides of triglyceridemia during which they move to the VLDL.

The patients with Tangier disease, a rare familial deficiency of HDL that was discovered at the NHLI in 1960, provide us with a unique opportunity to learn from an experiment of nature more about the functions of HDL. Last year, study of the small amounts of HDL obtainable from Tangier homozygotes had led to the conclusion that it lacked the apoprotein A-I. More recently, however, it was found using immunochemical methods that there is no selective reduction in the total amount of A-I (relative to A-II) in the plasma of these patients. Although we cannot rule out the possibility that the Tangier A-I is abnormal, the data available at present yield no evidence of differences between the composition of Tangier A-I (or A-II) and that of normals. Thus, all of the findings to date are consistent with the hypothesis that Tangier disease is the result of a regulatory rather than a structural gene defect.

During the past year the abnormalities of the low density lipoprotein (LDL) and VLDL previously noted in the Tangier patients have been investigated in detail. All of the four homozygotes studied had LDL of grossly abnormal lipid composition, undoubtedly secondary to the HDL deficiency. It appears that, in the absence of HDL, LDL does not function normally as a carrier of cholesterol. Its cholesterol content is low and triglyceride content markedly elevated. Preliminary experiments suggest that such triglyceride-rich LDL can serve as a substrate for lipoprotein lipase and this may account for the low levels of LDL in Tangier plasma. The VLDL of Tangier homozygotes, although morphologically and chemically normal, may exhibit an altered electrophoretic mobility. This was found to be attributable to a relative deficiency of the C apoproteins. When VLDL synthesis was increased by carbohydrate feeding, the C protein content of the VLDL was remarkably low in all four patients. On a normal dietary regimen, however, the C protein, although low in the two male patients, was normal in the two women. Perhaps this is related to the fact that HDL concentrations are normally higher in women than in men (this is one of the few known sex differences in plasma lipoproteins). In any case, all of the observations in the Tangier patients are consistent with the hypothesis that the HDL functions as a reservoir or sink for the C apoproteins that move between it and VLDL. Whether the accumulation of lipids in nerve and other tissues in Tangier disease is due to impaired removal of cholesterol secondary to the deficiency of HDL or to endocytosis of the abnormal circulating lipoproteins that are found in this disorder remains to be determined.

Further information on the movement of the C apoproteins between HDL and VLDL has been obtained by quantifying the changes in plasma lipoprotein distribution and composition following heparin-induced lipolysis in patients with hypertriglyceridemia. It appears that the C proteins preferentially associate with triglyceride-rich VLDL and accumulate in HDL only when the amount of VLDL and its associated triglyceride is markedly reduced. The data from these studies further suggest that the capacity of the HDL "reservoir" for C proteins may be relatively limited.

The partition of C proteins among the lipoproteins of different density classes is apparently distorted in several types of diseases. Because of the unique distribution of C apoproteins in patients with biliary cirrhosis the characteristic abnormal lipoprotein (LP-X) found in these patients is of particular interest. Until recently no combination of conventional separation methods had yielded preparations free of contamination with B apoprotein. Using zonal block electrophoresis, however, we have recently obtained preparations of LP-X that should be suitable for definitive analysis of its apoprotein content.

Certain important kinds of studies of lipoprotein metabolism can be carried out only with experimental animals or with isolated organ systems. The rat is a convenient subject for such work and characterization of the rat plasma lipoproteins was therefore undertaken several years ago. Last year it was reported that homologues for all of the major human apolipoproteins had been identified, purified and partially characterized. Significant quantitative differences between the rat and the human in terms of the distribution of individual apoproteins in lipoproteins of different densities have now been

defined. Studies of the apoproteins in the plasma and in the liver liposomes of rats fed orotic acid have enabled us to learn in which lipoprotein form the several C apoproteins are released from the liver and have led to the conclusion that the absence of one sialylated form of apoC-III in human abetalipoproteinemia reflects the failure of the liver to elaborate VLDL rather than a defect in sialylation.

It has been shown in this Institute and elsewhere that patients with type II hyperlipoproteinemia (familial hypercholesterolemia) have a defect in the feed-back regulation of cholesterol synthesis. This defect which can be demonstrated in skin fibroblasts cultured from affected individuals consists in a failure of the cells to respond to the presence of LDL with a decrease in the activity of hydroxyl-methylglutaryl coenzyme A (HMG CoA) reductase, the enzyme that catalyzes the rate-limiting step in cholesterol synthesis. Thus far, cultured fibroblasts from five patients homozygous for type II hyperlipoproteinemia have been studied; seven others have been initiated. In order to define further the mechanisms through which HMG CoA reductase activity is regulated - in normal and in type II cells - it will be necessary to measure the amounts of enzyme protein as well as enzyme activity in future studies. A highly purified HMG CoA reductase has been prepared by affinity chromatography and it is hoped that an antibody developed against this material will be usable for the immunoassay of the reductase protein. The purified reductase will also be used for investigation of the mechanisms through which its activity is apparently regulated rapidly and reversibly, perhaps by phosphorylation and dephosphorylation.

The type II Coronary Intervention Study, designed to test the hypothesis that reducing the concentration of plasma LDL can favorably affect the progression of established coronary artery disease, has been in progress for three years. In this study 250 suitable patients with type II hyperlipoproteinemia and coronary artery disease demonstrated by angiography, will be treated with dietary control alone or with diet plus cholestyramine and followed in a double-blind fashion for 2 to 5 years. Of the total of about 15,000 patients that have been referred for consideration for this study, 47% have been referred in the past year. There are at present 98 patients in the study, 54 who have completed their one year evaluations and 35 who have completed their two year evaluations. It is expected that a number of changes that have been made in the programs for referral and screening will expedite enrollment of patients in the study and make it possible to begin during the coming year to analyze some of the extensive data that have already been collected.

Project No. Z01 HL 02001-15 MDB
1. Molecular Disease Branch
2. Section on Lipoprotein Structure
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Lipoproteins of Tangier Disease

Previous Serial Number: NHLI-288(c)

Principal Investigators: Peter N. Herbert, M.D.
Robert J. Heinen, B.S.
Eve C. Church, M.S.

Other Investigators: None

Cooperating Units: Trudy Forte, Ph.D., Donner Labs., Berkeley, California
Victor J. Ferrans, M.D., NHLI
Donald S. Fredrickson, M.D., Institute of Medicine, National Academy of Sciences, Washington, D.C.

Project Description:

Objectives:

Tangier disease, a familial deficiency of high density lipoprotein (HDL), should provide unique insight into the obscure physiological role of plasma high density lipoproteins. This disorder represents a natural biochemical ablation experiment. Affected subjects demonstrate impressive storage of cholesteryl esters in reticuloendothelial cells and transient or severe peripheral neuropathy. This project was designed to elucidate the relationships between the plasma HDL deficiency, the abnormalities of plasma lipoproteins and the observed pathology.

Methods:

Large quantities of plasma were obtained by plasmapheresis from patients homozygous for Tangier disease. The methods of lipoprotein and apolipoprotein isolation and chemical analyses have been described in previous annual reports.

Major Findings:

1) The beta (rather than the normal alpha-2) electrophoretic mobility of Tangier very low density lipoproteins (VLDL) was shown to be attributable to a relative, though not absolute, C-apoprotein deficiency, a defect

paralleling that found in type III hyperlipoproteinemia. A peculiar, apparently sex and diet related, variation in C-protein composition was observed. When VLDL synthesis was induced by carbohydrate feeding all four Tangier homozygotes studied had remarkably low VLDL C-protein content - 10-30% (normal 40-50%). The two female patients, in contrast to the males, had a normal C-protein content on normal or high fat diet. These findings confirm the supposition that HDL serves as an important reservoir for the C-apoproteins but suggests that significant sex differences in C-protein metabolism may exist.

2) All Tangier homozygotes studied had low density lipoproteins (LDL) of grossly abnormal chemical composition. Triglyceride was increased from the normal 6-10% to 30-35% and cholesterol decreased to 15-20% (normal 38-45%). This abnormality of LDL composition undoubtedly is related to the HDL deficiency and suggests that cholesteryl esters of HDL origin normally exchange for triglyceride in the metabolic conversion of VLDL to LDL. In the absence of HDL, LDL does not serve its normal role as the repository of plasma cholesterol. The very high triglyceride content of Tangier plasma LDL, in addition, may account for the low levels of LDL characteristic of this disorder since preliminary experiments suggest that such triglyceride rich LDL can serve as a substrate for lipoprotein lipase.

3) The lipid accumulation in Tangier tissues has been evaluated by light and electron microscopic studies of bone marrow, skin and gastrointestinal tissues from affected subjects. Most striking was the accumulation of neutral lipid in Schwann cells and even nerve axons, a finding undoubtedly related to the peripheral neuropathy in these patients. It is not known whether this lipid arises from endocytosis of abnormal circulating lipoproteins or reflects a failure of HDL to remove lipid from nerves by net transfer. The former mechanism gains support from the demonstration of bizarre lipoprotein forms in Tangier HDL which appear to be generated during chylomicron catabolism and which disappear when the diet contains no fat.

4) Immunochemical studies of Tangier plasma failed to support earlier claims that the A-I apoprotein content was selectively reduced. Preliminary studies have failed to demonstrate compositional differences between Tangier and normal A-I and A-II. If this finding is confirmed when Tangier A-I and A-I are isolated in quantity, the data will point toward a regulatory rather than structural genetic defect in this disorder.

Significance to Biomedical Research and the Program of the Institute:

HDL concentrations are higher in women than in men, one of the few lipoprotein differences between the two sexes. HDL concentrations are also very high in species resistant to atherosclerosis. The functions of HDL are only partially known. Study of Tangier disease offers unique opportunities for determining the structure and function of all lipoproteins.

Proposed Course:

1) The lipid and apoprotein composition of the abnormal lipoproteins

generated during chylomicron catabolism in Tangier disease will be defined.

2) The plasma lipoproteins of Tangier heterozygotes will be examined on normal diets and after carbohydrate feeding using the techniques applied in the studies of the Tangier homozygotes.

3) Tangier apoA-I and apoA-I will be isolated and characterized chemically and immunologically to resolve the issue of a structural defect in this disorder.

Publications:

1. Ferrans, V. J., and Fredrickson, D. S.: Pathology of Tangier Disease. Amer. J. Path. 78: 101-136, 1975.

Key Words:

1) lipoproteins
2) apolipoproteins
3) HDL - high density lipoproteins
4) VLDL - very low density lipoproteins
5) LDL - low density lipoproteins
6) atherosclerosis
7) lipoprotein deficiencies
8) dyslipoproteinemias
9) hyperlipoproteinemia
10) Tangier disease

Project No. Z01 HL 02007-08 MDB
1. Molecular Disease Branch
2. Section on Lipoprotein Structure
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Characterization of the Human Plasma Apolipoproteins

Previous Serial Number: NHLI-296 and NHLI-298(c)

Principal Investigators: Peter N. Herbert, M.D.
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J. Roger Lee, B.A.
Marguerite J. LaPiana, B.A.
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Richard S. Shulman, M.D.

Other Investigators: None

Cooperating Units: Trudy Forte, Ph.D., Frank Lindgren, Ph.D., and Alexander Nichols, Ph.D., Donner Laboratories, Berkeley, California

Project Description:

Objectives:

Studies in recent years have revealed that abnormalities of plasma lipid concentrations are frequently associated with altered distributions of the individual apolipoproteins among different density classes of lipoproteins. It is not known whether these are cause or effect relationships. This project is part of a continuing effort to characterize the major and minor apolipoproteins and to relate their distribution to various types of well-defined clinical disorders.

Methods:

Plasma was obtained from patients with types I, II, III and V hyperlipoproteinemia, biliary cirrhosis and familial homozygous hypobetalipoproteinemia. The techniques of lipoprotein isolation by ultracentrifugation and agarose column chromatography, and apolipoprotein purification by rapid-flow-gel and ion-chromatography have been described in previous annual reports. Preparative polyacrylamide gel electrophoresis has been added to the immunochemical and electrophoretic techniques employed in earlier studies.

Major Findings:

1) Investigation of the apoprotein content of the β-VLDL of type III

subjects has continued. It was reported last year that the C-protein contribution to type III VLDL was reduced by approximately 20%. The apoproteins from the VLDL of several additional patients have been fractionated and this conclusion seems partially inaccurate. Three patients with type III had VLDL C-protein contents within the normal range and, of greater interest, the VLDL had normal electrophoretic mobility. This finding underscores the unreliability of electrophoresis in lipoprotein phenotyping and supports our contention that the cholesterol content of type III VLDL is a more specific marker in this disorder. In addition, the published report that type III VLDL is disproportionately rich in the "high-arginine" apolipoprotein was not confirmed.

2) The lipoproteins and apolipoproteins from two subjects homozygous for the gene of hypobetalipoproteinemia were isolated and compared with normals and with a subject having the more common autosomal recessive abetalipoproteinemia. The VLDL and LDL fraction from the one homozygous hypobetalipoproteinemic (H-H) subject extensively evaluated contained no immunochemical traces of beta lipoprotein, but did contain apoA-I, apoA-II and the C-apoproteins. Barrel-shaped structures identical to those seen in the LDL fraction of the common variety of abetalipoproteinemia were observed on electron microscopy. Polyacrylamide gel electrophoresis of apoHDL from both H-H subjects demonstrated reduction but not complete absence of the C-III-1 apoprotein, a finding previously reported in abetalipoproteinemia.

3) Studies of the effects of in vivo heparin-induced triglyceride hydrolysis on the distribution of the C-apoproteins were completed. One subject was studied twice, at plasma triglyceride concentrations of 2700 mg% and 2400 mg%, and triglycerides fell 45% and 25% respectively in response to heparin injection. The C-protein content of the VLDL, however, was unchanged after lipolysis in both experiments and in neither experiment did the VLDL hydrolysis lead to significant increases in the $S_f^o$ 0-20 lipoprotein class. Two additional subjects were studied at triglyceride levels of 364 mg% and 426 mg%. Their plasma triglycerides fell by 64% and 53% with heparin-induced lipolysis. In contrast to the patient with higher triglyceride levels, accumulation of lipoprotein in the $S_f^o$ 0-20 class was observed and decreases of 47 and 64% in the total VLDL protein occurred. One patient showed no change in the relative C-protein content in VLDL while the second had a 50% drop. There was an 8% increase in the HDL C-protein in the latter subject. These results suggest that over a wide range of plasma triglyceride concentrations the C-proteins selectively associate with the triglyceride-rich VLDL and redistribute to HDL only when VLDL (and triglyceride) concentrations are drastically reduced. They also suggest that HDL may have a limited capacity to accommodate C-proteins after VLDL lipolysis.

4) Work has progressed on the primary structure of apoC-II. In contrast to the two C-proteins which have been sequenced, apoC-II presents special problems in obtaining adequate quantities for this analysis. Nevertheless, enzymatic and chemical peptides have been generated from this apoprotein and the sequential degradation and alignment of these peptides is about 75% complete. In contrast to the intact apoprotein, none of these peptides has

been found capable of lipoprotein lipase activation.

5) The LP-X lipoprotein of primary biliary cirrhosis is being investigated because of the unique redistribution of C-apoproteins which characterizes this disorder. No combination of preparative ultracentrifugation and agarose gel chromatography satisfactorily resolved the LP-X lipoprotein from contamination with LDL containing B-apoprotein. A system of zonal block electrophoresis has been developed which appears capable of this separation. Preliminary results suggest that all previous estimates of the quantitative and qualitative protein content of the LP-X lipoprotein were in error.

Significance to Biomedical Research and the Program of the Institute:

The apolipoprotein serves a critical role in the stabilization of plasma lipids, provides co-factor activities for lipolysis and may well modulate the fine controls that govern plasma lipoprotein concentrations. The characterization should contribute greatly to our understanding of physiological and pathological lipoprotein metabolism.

Proposed Course:

1) The characterization of the lipid and apolipoprotein distributions in type III hyperlipoproteinemia and primary biliary cirrhosis will be completed.

2) Current critical analyses of the available preparative techniques for lipoprotein and apolipoprotein purification will be continued.

3) The primary structure determination of apoC-II will be finished.

4) Isolation and characterization of the various "arginine-rich" apolipoproteins will continue with an effort to relate their occurrence and distribution to abnormalities of plasma lipid transport.

Publications:

1. Brewer, H. B., Jr., Shulman, R. S., Herbert, P. N., Ronan, R., and Wehrly, K.: The complete amino acid sequence of alanine apolipoprotein (apoC-III), an apolipoprotein from human plasma very low density lipoproteins. J. Biol. Chem. 249: 4975-4984, 1974. (Cited as "in press" in 1974 project report NHLI-296).

2. Shulman, R. S., Herbert, P. N., Wehrly, K., and Fredrickson, D. S.: The complete amino acid sequence of C-I (apoLp-Ser), an apolipoprotein from human very low density lipoproteins. J. Biol. Chem. 250: 182-190, 1975. (Cited as "in press" in 1974 project report NHLI-296).

3. Herbert, P. N., Forte, T. M., Shulman, R. S., LaPiana, M. J., Gong, E. L., Levy, R. I., Fredrickson, D. S., and Nichols, A. V.: Structural and

compositional changes attending the ultracentrifugation of very low density lipoproteins. Prep. Biochem., in press.

Key Words:

1) lipoproteins
2) apolipoproteins
3) HDL - high density lipoproteins
4) VLDL - very low density lipoproteins
5) LDL - low density lipoproteins
6) atherosclerosis
7) lipoprotein deficiencies
8) dyslipoproteinemias
9) hyperlipoproteinemia
10) abetalipoproteinemia

Project No. Z01 HL 02008-06 MDB
1. Molecular Disease Branch
2. Section on Lipoprotein Structure
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Rat Plasma Lipoproteins and Apolipoproteins

Previous Serial Number: NHLI-297

Principal Investigators: Peter N. Herbert, M.D.
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Other Investigators: Marguerite J. LaPiana, B.A.
J. Roger Lee, B.A.
John Stonik, B.S.

Cooperating Units: Herbert G. Windmueller, Ph.D., Laboratory of Nutrition & Endocrinology, NIAMDD

Project Description:

Objectives:

The study of lipoprotein metabolism and interrelations is most conveniently undertaken in non-human species were isolated organ systems are available and extensive perturbation is possible. The rat has been widely employed for such studies, but rat lipoproteins and apolipoproteins remain incompletely characterized. Such characterization of the rat apolipoproteins has been the subject of study in this laboratory for the last six years. Employing orotic acid fed rats, studies have commenced regarding the lipoprotein origins of the apolipoproteins.

Methods:

The techniques of lipoprotein isolation and apolipoprotein fractionation and characterization have been outlined in previous annual reports. In addition, hepatic liposomes from orotic acid fed rats have been isolated as described by others (J. Lipid Res. 12: 450-459, 1970).

Major Findings:

1) As detailed in the 1974 project report, homologues of all of the human apolipoproteins have been identified in the rat and purified to homogeneity. They have been characterized by amino acid analysis, COOH and $NH_2$ terminal residues, carbohydration and molecular weight by gel chromatography

2) Significant quantitative differences between the distribution of apolipoproteins in the human and rat were identified.

a) The C-protein group was shown to constitute 20% of the mass of rat high density apolipoprotein contrasted to 10% in the human.

b) The C-II apolipoprotein, the activator of lipoprotein lipase in the rat and human, was found in higher concentration in rat HDL than in VLDL. This relative distribution is reversed in the human.

c) The arginine-rich apolipoprotein was found to comprise 10-15% of the total rat apoHDL, whereas its human counterpart is found in only trace amounts in this density class.

d) The A-II apoprotein which constitutes 20-30% of human apoHDL was a relatively minor component ( < 5%) of rat apoHDL.

It remains to be demonstrated whether these differences in apolipoprotein distribution can be related to the well-known differences in plasma lipoprotein metabolism in the rat and human.

3) Rats fed a diet containing 1% orotic acid develop hepatic steatosis and a plasma lipoprotein profile resembling that in patients with abetalipoproteinemia. The plasma and hepatic lipoproteins and apolipoproteins of these animals were extensively investigated. The plasma from 75% of the treated rats contained no immunochemical traces of the betalipoprotein after 7-14 days.

Studies of the plasma apolipoproteins and those associated with liver liposomes suggested that:

a) ApoC-I and apoC-II are secreted by the liver in association with both HDL and VLDL.

b) C-III-0 is secreted with VLDL and C-III-3 only with HDL.

c) The absence of one sialylated form of apoC-III (C-III-1) in human abetalipoproteinemia reflects failure of the liver to elaborate VLDL, rather than a defect in sialylation.

Significance to Biomedical Research and the Program of the Institute:

The completion of the studies described should greatly facilitate extrapolation of results of physiological experiments in the rat to problems of lipoprotein physiology and pathology in the human. The orotic acid fed rat, specifically, has already contributed to our knowledge of the lipoprotein origins of the apolipoproteins.

Proposed Course:

1) Characterization of rat apoLDL, arginine-rich protein and the apoproteins of all density classes in intestinal lymph will be completed.

2) The mechanism of the rat C-II apoprotein activation of lipoprotein lipase will be further defined.

3) The work on the orotic acid fed rat will be extended in studies of the apolipoprotein elaboration by the isolated perfused liver.

Publications:

1. Herbert, P. N., Windmueller, H. G., Bersot, T. P., and Shulman, R. S.: Characterization of the rat apolipoproteins. I. The low molecular weight proteins of rat plasma high density lipoproteins. J. Biol. Chem. 249: 5718-5724, 1974. (Cited as "in press" in 1974 project report NHLI-297).

Key Words:

1) rat
2) lipoproteins
3) apolipoproteins
4) HDL - high density lipoproteins
5) VLDL - very low density lipoproteins
6) LDL - low density lipoproteins
7) orotic acid
8) abetalipoproteinemia
9) organ perfusion
10) hepatic steatosis

Project No. Z01 HL 02002-03 MDB
1. Molecular Disease Branch
2. Section on Lipoproteins
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: NHLI Type II Coronary Intervention Study

Previous Serial Number: NHLI-289(c)

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John F. Brensike, M.D.
Eugene R. Passamani, M.D.
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Other Investigators:
Patricia Strobell
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Cooperating Units:
Cardiology Branch, NHLI
Lipid Metabolism Branch, NHLI
Data Management Branch, Division of Computer, NIH
Research and Technology, NIH
Biometrics Research Branch, NIH
Clinical Center Administration, NIH

Project Description:

Objectives:

The primary aim of this study is to determine whether lowering the LDL cholesterol in patients with premature coronary artery disease and type II will slow, stop or reverse the progression of coronary artery disease (i.e., to test the lipid hypothesis in a specific group of high risk patients).

Methods:

Two hundred-fifty patients with coronary artery disease demonstrated by coronary angiography who meet all the criteria outlined in the protocol will be randomly split into two equal groups (treatment - 24 grams cholestyramine and diet control - control - 24 grams placebo and diet control). They will



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be followed in a double blind fashion monthly for 2-5 years. Repeat coronary angiography will be performed at 2 years and at 5 years (if the study continues).

The end point will be: 1) a significant difference in the progression of coronary disease as shown on angiography, or 2) a significant difference in new myocardial infarctions or death, or 3) lack of any of the above at 5 years.

Major Findings:

1) To date (April 1, 1975) the NHLI Type II Coronary Intervention Study has had approximately 15,000 patients referred for consideration for the study. Of these, about 7,000 or 47% have been referred within the last year. At current rates of intake, it is estimated that 10,000 to 15,000 new patients will be screened within the next year.

Of the 15,000 patients referred, approximately 8,250 were not eligible for the program after the lab screening (they were not cholesterol eligible or had elevated triglycerides). Of the remaining 6,750, approximately 600 are pending further processing; of the 6,150 processed beyond this point, about 3,150 were found to be ineligible over the telephone. Of the remaining 3,000, 750 have not been interested in being considered for the program, and another 30% or 900 have been found to not meet the LDL requirements (not type II) and were therefore ineligible for the program.

The remainder (approximately 1,350) have either been seen (1,200) or are scheduled to be seen and evaluated in detail, including measurement of approximately 20 risk factors, determination of presence or absence of evidence for underlying coronary artery disease by history, physical and non-invasive testing, determination of dietary effect on their lipid abnormality and classification as type II.

Currently there are 98 patients in the program and on medication, 54 patients who have completed their year evaluation and 35 patients who have completed their two year evaluation.

Results to date include:

1) Less progression of coronary artery disease in study patients than expected and probable regression (an unexpected finding) in at least two patients.

2) Changes in angiography (better or worse) were seen in approximately 40% of the patients who have returned for their two year catheterization.

3) Improved or unchanged symptomatic status has been observed in all patients will in the program with objective improvement (exercise test, etc.) in many.

4) Four deaths and two documented MI's have occurred in patients in the program.

An additional year's experience has confirmed the preliminary observations made last year. Namely -

5) An approximate 10% incidence of positive exercise tests in asymptomatic type II's has been observed (about 3-5 times greater than that reported in the literature).

6) An approximate 13% incidence of coronary calcifications has been observed in asymptomatic type II's giving an overall incidence of approximately 20% of asymptomatic patients 21-55 with type II who have evidence of occult coronary artery disease (positive exercise test and/or coronary calcifications).

7) Approximately 40% of our patients have normalized on diet and in these the average cholesterol drop is 25%. The overall cholesterol drop is 13-15% including all patients (non-adherers as well as adherers).

8) Exercise testing does not appear to be as sensitive or specific in an asymptomatic population (even a high risk population like this one) as many previously thought and our data raise real questions about its usefulness in individual patient care decisions.

9) Coronary calcifications on fluoroscopy is a readily available, inexpensive procedure with little patient morbidity and appears to be a better screening procedure for occult coronary artery disease in our asymptomatic type II population than the more expensive and elaborate exercise testing.

10) Evaluation of a sub-group of our patients who were psychologically typed (type A vs. type B) does not substantiate the hypothesis that type A personalities are more likely to have coronary artery disease than type B personalities.

11) The Safety Monitoring Board has not informed us of any untoward or new reactions to the medication nor have we noted any reactions not previously described except for a few episodes of tinnitus.

Significance to Biomedical Research and the Program of the Institute:

The answer to the question posed by this study is one of the most important in the field of heart research. It has implications not only for those patients who are type II with coronary artery disease, but also for type II's without disease, and for the general population. Also the knowledge gained about the natural history of coronary artery disease and the factors affecting it will significantly advance our current knowledge and have implications for the treatment of the major cause of mortality in the adult population of the United States.

Proposed Course:

1) Our major effort will continue to be to expedite patient enrollment into the program. This is possible because of changes which have been made in the program office. Seven months ago it was decided that, in the interest of completing the program in a reasonable period of time and of ensuring the quality and accuracy of the essential information for the program, a) some of the individualized attention which patients had formerly received would have to be sacrificed (realizing that this might increase patient dropouts) and b) some of the patient's service and information of lesser scientific importance (diet information, general patient laboratory and history information) would have to be dropped in order to allow us to process two to three times the number of patients which we were currently seeing with the same personnel.

Over the last seven months, this new system has evolved and is currently being used. Approximately twice the number of people are being processed and it appears to be working. Our proposed course is to continue to expand the system as much as outside facilities allow to the limit of approximately three times previous enrollment.

2) In addition, where possible, we will attempt to make our referrals more selective and will begin to analyze and process the extensive results obtained to date. Currently, we have 98 people in the program, 35 of whom have received re-catheterization at two years. These 35 individuals have had over 30 risk factors measured on them periodically, as well as the invasive and non-invasive cardiovascular tests performed. In addition, we have 54 people who have been through their one year evaluation and have documented coronary artery disease, as well as repetitive measurement of over 30 risk factors and baseline invasive and non-invasive test information. Finally, we have approximately 1,050 patients with type II who have been studied in detail with over approximately 20 risk factors measured and pre- and post-diet information available.

Publications:

None

Key Words:

1) type II
2) coronary artery disease
3) LDL cholesterol
4) angiography
5) intervention study
6) catheterization
7) cardiovascular tests
8) high risk patients
9) epidemiology
10) double blinded trial

Project No. Z01 HL 02003-05 MDB
1. Molecular Disease Branch
2. Section on Lipoproteins
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

| | |
|---|---|
| Project Title: | The Biochemistry and Metabolism of Plasma Lipoproteins |
| Previous Serial Number: | NHLI-291(c) |
| Principal Investigators: | Robert I. Levy, M.D.<br>Conrad B. Blum, M.D. |
| Other Investigators: | Leslie Jenkins, M.A. |
| Cooperating Units: | Frank T. Lindgren, Ph.D., University of California, Berkeley, California<br>Mones Berman, Ph.D., NCI<br>Marshall Hall, M.D., NCI<br>Shlomo Eisenberg, M.D., Hadassah Medical Center, Jerusalem, Israel |

Project Description:

Objectives:

1) To determine the metabolic behavior of $^{125}$I-VLDL in various types of hyperlipoproteinemia under basal conditions and under various metabolic and nutritional perturbations.

2) To establish the metabolic behavior of plasma HDL, extending the already developed multicompartmental model of apolipoprotein metabolism to include the two major apoproteins of HDL.

3) To elucidate the factors involved in exchange and equilibrium of C-peptides between VLDL and HDL.

Methods:

The isolation, purification, and iodination of VLDL, LDL, and HDL have been described previously. The VLDL, LDL, and HDL apoproteins are separated electrophoretically on 15% polyacrylamide gels using a continuous buffer system with 0.1% sodium lauryl sulfate.

Major Findings:

Methodology for performing $^{125}$I-HDL turnover studies has been developed

and eight studies have been completed in five normal volunteers. Another seven studies are underway and should be completed by July 1, 1975. A number of insights into high density lipoprotein metabolism have resulted: (a) the two major peptides of high density lipoprotein seem to be metabolized homogeneously in contrast to the B and C peptides of VLDL; (b) the kinetics of high density lipoprotein and its two major peptides approximate a simple two-compartmental model; (c) synthesis rates and fractional metabolic rates for HDL protein have been computed for normal and perturbed conditions, showing that (d) the fall in HDL levels accompanying carbohydrate feeding is solely a result of altered catabolism with synthesis remaining constant.

Significance to Biomedical Research and the Program of the Institute:

Since the predominant protein of HDL is the activator for lecithin-cholesterol acyl transferase and HDL is the natural substrate for this enzyme, a knowledge of the biological behavior is crucial to a full understanding of cholesterol metabolism. The hypothesis of some that HDL is responsible for removal of cholesterol from tissues heightens interest in its biological behavior.

Furthermore, since HDL acts as a reservoir for the C-peptides (including apoC-II, the activator for lipoprotein lipase) it plays an important role in regulating triglyceride metabolism.

Proposed Course:

1) Studies of VLDL metabolism will continue, especially focusing on effects of nutritional and pharmacologic perturbations.

2) Studies of the turnover of HDL peptides will be extended to include additional perturbations in testing and further developing the multi-compartmental modeling.

Publications:

1. Blum, C., and Levy, R. I.: Interconversions of apolipoprotein fragments. Ann. Rev. Med., in press.

2. Eisenberg, S., and Levy, R. I.: Lipoprotein metabolism. Advan. Lipid Res., in press.

Key Words:

1) VLDL - very low density lipoproteins
2) LDL - low density lipoproteins
3) HDL - high density lipoproteins
4) apoproteins
5) turnover studies
6) $^{125}I$

Project No. Z01 HL 02004-08 MDB
1. Molecular Disease Branch
2. Section on Genetics and Lipid Biochemistry
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Lipid Constituents of Human Tissues

Previous Serial Number: NHLI-293(c)

Principal Investigators: Howard R. Sloan, M.D., Ph.D.
Gerd Assmann, M.D.

Other Investigators: Barbara Davis, M.S.
Stephen Demosky, B.S.
Anne Flory, B.S.

Cooperating Units: None

Project Description:

The principal objective of this study is the development of inclusive methods for the determination of simple and complex lipids, including the neutral glycolipids, the gangliosides, the plasmalogens, complex sterols and steryl esters, and partial glycerides in plasma and tissues, for purposes of improved diagnosis of the already established lipidoses and evaluation of heretofore unknown lipid storage diseases. The human liver has been used as the prototype tissue source; the spleen, lymph nodes and adrenal, however, have also been studied. The methodology is particularly needed in order to gain an insight into the biochemical interrelationships of each of these lipid classes and sub-classes with each other and with the other major lipid classes.

The methods of extracting, separating and analyzing the various lipids in human tissues have been thoroughly described in PHS-NIH Individual Project Report, July 1, 1973 through June 30, 1974, Serial No. NHLI-293(c). New methods, including conversion to acetylated derivatives, have been developed for the identification and quantification of the carbohydrate components of lipids. In addition, methods have been developed for the identification of several new lipids employing the techniques of gas-liquid-mass spectrometry and high resolution mass spectrometry as well as liquid-liquid chromatography.

Techniques have been developed for analyzing all of the major lipid classes and sub-classes in human tissue. The normal values and ranges for these lipids have been presented in a prior annual report (NHLI-264[c]). The use of the techniques developed in this study have permitted the post-abortion confirmation of the in utero diagnosis of one case of Niemann-Pick disease, type A (in addition to the three cases correctly diagnosed last year). Two cases of $G_{M1}$ gangliosidosis have been confirmed by chemical examination of

the aborted tissues as well as an additional case of metachromatic leukodystrophy (total now three).

Analyses of post-mortem samples of liver, spleen and adrenals of two more patients with Wolman's disease (in addition to the one studied last year) revealed the accumulation of the following sterols: 7-alpha-hydroxycholesteryl ester, 7-beta-hydroxy-, 7-keto-, 5-6-alpha-epoxy- and 5-6-beta-epoxy-cholesteryl esters. These compounds were isolated and identified by gas chromatography, mass spectroscopy, and NMP spectrometry.

Tissue storage of lipids is a principal process in atheromata formation. Studies which elucidate abnormalities in lipid metabolism and provide new means for the detection of biochemical defects offer useful new approaches which may yield specific clues to the process of atherogenesis. Investigation of tissues, particularly liver, spleen and adrenal, from patients who have died of previously unclassified lipidoses is in progress.

Publications:

1. Kaback, M. M., Sloan, H. R., Sonneborn, M., Herndon, R. M., and Percy, A. K.: $G_{M1}$-gangliosidosis type I: in utero detection and fetal manifestations. J. Pediat. 82: 1037-1041, 1973.

2. Assmann, G., Fredrickson, D. S., Sloan, H. R., Fales, H. M., and Highet, R. J.: Accumulation of oxygenated steryl esters in Wolman's disease. J. Lipid Res. 16: 28-38, 1975.

Key Words:

1) lipids
2) human
3) tissues
4) neutral lipids
5) phospholipids
6) glycolipids
7) sphingolipids

Project No. Z01 HL 02005-07 MDB
1. Molecular Disease Branch
2. Section on Genetics and Lipid Biochemistry
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Tissue Lipidoses and Hyperlipoproteinemias

Previous Serial Number: NHLI-294(c)

Principal Investigators: Howard R. Sloan, M.D., Ph.D.
Gerd Assmann, M.D.

Other Investigators: Barbara Davis, M.S.
Stephen Demosky, B.S.
Briston Williamson
Arthur Pace

Cooperating Units: None

Project Description:

The principal objective of this study is to improve the knowledge of the biochemical and the enzymatic basis of the genetically determined tissue lipid storage disorders as well as to improve diagnostic techniques for both the patients and possible heterozygous carriers. Findings in this and other laboratories have demonstrated that tissue culture cells derived from the bone marrow and skin of patients with all of the lipid storage diseases have the same genetic profile as the patient from whom they were obtained. It is, therefore, possible to diagnose all of these diseases by the appropriate enzymatic analysis of cells derived from tissue culture of the skin rather than by resorting to biopsy of the liver or some other organ. The study of these diseases offers a model of great interest for a study of the storage of lipid within tissues.

The discovery of the new technique of cell hybridization has opened an additional path for the study of these inherited metabolic disorders. Hybrids of particular interest include: hybrid cell lines produced by crosses between fibroblast-like cells from patients with different forms of Niemann-Pick disease, different forms of Gaucher's disease, and different forms of $G_{M1}$ gangliosidosis. Hybrids are also being prepared with cells from individuals with the various lipid storage diseases and with mouse L-cells. Additional hybrids between mouse L-cells and fibroblasts derived from patients with homozygous type II and type III hyperlipoproteinemia have been produced.

In annual report NHLI-294(c) the methods of obtaining, processing, storing and placing into culture tissues from patients with the various lipid storage disorders and the hyperlipoproteinemias have been described. In addition, methods have been developed for assaying and purifying several of the enzymes

that catabolize sphingolipids. These enzymes are present in decreased amounts in the sphingolipidoses.

Normal ranges for many of the enzymes involved in the catabolism of the sphingolipids have been established for normal human fibroblasts and fibroblasts obtained from normal amniotic fluid. It is now possible to make a diagnosis (including a pre-natal diagnosis) of all of the lipid storage diseases. Many successful pre-natal diagnoses have been made and no false positives or false negatives have been obtained.

Cholesteryl ester hydrolase (acid) has been purified over 1,000-fold. Minimal amounts of triglyceride lipase activity remain in these preparations and two bands are seen on polyacrylamide gel electrophoresis. More than 99.9% of the triglyceride lipase activity has, however, been removed.

Cell lines from 12 patients with homozygous type II hyperlipoproteinemia and 10 patients with type III hyperlipoproteinemia have been initiated. In five of the type II lines serum causes an insignificant decrease in HMG CoA reductase activity compared with normal cell lines. It has been observed that when some cells are treated with the detergent Kyro EOB, significant amounts of HMG CoA hydrolase are released, leading to the formation of acetoacetic acid and then acetone during the course of the reductase assay. The formation of the latter product has been conclusively demonstrated by classical organic chemical techniques. The release of the undesired HMG CoA hydrolase can be avoided by extracting the enzyme from the tissue culture cells in the following manner. The temperature of a suspension of the cells is gradually lowered from +5°C to -50°C at a rate of 5°C per minute. The tube containing the cell suspension is then placed in air at 25°C and allowed to thaw slowly. This procedure is repeated twice.

Tissue storage of lipids is a principal process in atheroma formation. Studies which elucidate abnormalities in lipid metabolism and which provide new means for the differentiation of biochemical defects offer useful new approaches which may yield specific clues to the process of atherogenesis. Moreover, a perpetuation of metabolic disorders in itssue culture cells makes possible a detailed approach to the pathogenesis and control of metabolic disorders. In addition, this perpetuation may facilitate the study of the genetic defect in the various lipid storage disorders. The purification of the various sphingolipid hydrolases may permit detailed understanding of several lipid storage disorders at the molecular level, and thereby facilitate our understanding of the process of lipid accumulation within tissues.

Future studies will be directed at attempting to delineate differences in cholesterol metabolism between normal cultured cells and those derived from patients with the various hyperlipoproteinemias. The differential effects of serum, plasma, and various fractions of lipoproteins on the activity of HMG CoA reductase will be evaluated in these cell lines. In addition, detailed studies of HMG CoA reductase activity in the various hybrids will be conducted in an attempt to determine the nature, and possibly the chromosomal location, of those factors that are responsible for the normal feedback inhibition

exerted by cholesterol on HMG CoA reductase. Similar studies will be employed to differentiate more clearly the differences between homozygous type II hyperlipoproteinemia and type III hyperlipoproteinemia.

Publications:

1. Breslow, J. L., Lux, S. E., Spaulding, D. R., and Sloan, H. R.: 3-hydroxy-3-methyl glutaryl coenzyme A reductase activity in fibroblasts from phenotypic homozygous type II hyperlipoproteinemia. Pediat. Res. 8: 386-388, 1974.

Key Words:

1) lipid storage disorders
2) lipoproteins
3) hyperlipoproteinemias
4) tissue culture
5) hybridization
6) enzymes of lipid metabolism
7) HMG CoA reductase
8) cholesterol biosynthesis
9) fibroblasts

Project No. Z01 HL 02006-03 MDB
1. Molecular Disease Branch
2. Section on Genetics and Lipid Biochemistry
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

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|---|---|
| Project Title: | Microscopic Studies in Tissue Lipid Storage Diseases |
| Previous Serial Number: | NHLI-295(c) |
| Principal Investigators: | Howard R. Sloan, M.D., Ph.D. |
| Other Investigators: | Stephen Demosky, B.S.<br>Briston Williamson |
| Cooperating Units: | Victor J. Ferrans, M.D., Ph.D.<br>Section of Pathology, NHLI |

Project Description:

Microscopic study of tissues obtained from patients with tissue lipid storage disorders can contribute to the understanding of the biochemical basis of these genetically determined diseases and to the improvement of diagnostic techniques which may benefit potential carriers as well as those afflicted carriers. These techniques can also help to elucidate the nature of the cellular abnormalities in the hyperlipoproteinemias. The light and electron microscopic appearance of these macrophages may provide clues to the processes by which lipid is stored within tissues in pathological conditions.

To obtain tissues for these studies, patients with lipid storage diseases and hyperlipoproteinemias are admitted for evaluation including biopsy of the bone marrow and/or liver. Tissues are fixed in Baker's formalin, phosphate-buffered glutaraldehyde, and digitonin-glutaraldehyde and prepared for examination with light and electron microscopes.

We have now completed electron microscopic examination of fibroblasts derived from patients with various lipid storage diseases and the hyperlipoproteinemias, including patients who are homozygous for type II hyperlipoproteinemia. There are significant differences between the different diseases and an initial subclassification now seems possible.

Significant differences have been demonstrated at the electron microscopic level between the lipid-laden macrophages observed in various forms of Niemann-Pick disease. These differences do not at this time, however, permit a definitive diagnosis. The macrophages from patients with Gaucher's disease, $G_{M1}$-gangliosidosis and Tangier disease are clearly distinguishable

on the basis of appearance in electron micrographs. Study of the macrophages from patients with various hyperlipoproteinemias suggest that at least a tentative diagnosis of Tangier disease and possibly of type III hyperlipoproteinemia could be made in this way.

Auto-fluorescence analysis of macrophages in various tissue lipid storage diseases demonstrates the presence of fluorescent material in almost all of the lipid storage diseases. It is not possible at this time to make a definitive diagnosis of any of the lipid storage diseases based on the auto-fluorescence spectrum obtained from examination of the lipid laden macrophages. Significant difference between tissues from patients with the various lipid storage diseases have, however, been demonstrated.

In other studies, cultured cells were grown on cover slips and then incubated with 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (BCI-gal). Cultures of peripheral blood leukocytes and thick sections (10 μ) of human liver, spleen and kidney were also incubated with this substrate. The indigo produced following cleavage of the terminal galactose of this molecule makes it possible to visualize the subcellular localization of several β-galactosidases. Use of the BCI-gal assay has enabled us to make the intrauterine diagnosis of five cases of $G_{M1}$-gangliosidosis, including the first and second cases. Chemical, enzymatic, microscopic, and histochemical studies of tissues from the abortuses have verified the diagnoses. The technique has also been utilized to predict that twelve fetuses, known to be at risk for $G_{M1}$-gangliosidosis, would be normal. The clinical course of the twelve children adjudged by histochemical and enzymatic tests to be normal, have verified the in utero diagnosis.

Tissue storage of lipids is a principal process in atheroma formation. Light microscopic and electron microscopic studies may help to elucidate the mechanism by which lipid is stored within tissues in various pathological conditions and may provide specific clues to the study of the process of atherogenesis. The differences already noted may help to explain the differences between several different types of atheromata formation.

These further electron microscopic studies of cells and tissues from patients with the lipid storage disorders are essentially completed and will be terminated when all samples presently on hand have been examined.

Publications:

1. Sloan, H. R., and Breslow, J.: Foam cells. In Hematology of Infancy and Childhood (Nathan, D. and Oski, F., eds.), Saunders, Phila., 1974, pp. 761-780. (Cited as "in press" in project report NHLI-295(c), 1974.

Key Words:

1) light microscope
2) electron microscope
3) lipid storage disorders
4) hyperlipoproteinemias
5) *in utero* diagnosis
6) lipid-laden macrophages
7) auto-fluorescence

Project No. Z01 HL 02009-08 MDB
1. Molecular Disease Branch
2. Section on Peptide Chemistry
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

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|---|---|
| Project Title: | Structure and Function of Parathyroid Hormone |
| Previous Serial Number: | NHLI-299 |
| Principal Investigators: | H. Bryan Brewer, Jr., M.D.<br>Thomas Fairwell, Ph.D. |
| Other Investigators: | Rosemary Ronan, B.A.<br>Ann LaRue, B.A. |
| Cooperating Units: | Claude Arnaud, Mayo Clinic, Rochester, Minn.<br>Werner Rittel, Ciba Geigy Pharmaceutical Co., Basle, Switzerland |

Project Description:

1) Objective:

Isolation, characterization, and sequence analysis of human parathyroid hormone(HPTH).

Methods Employed:

HPTH has been isolated from parathyroid adenomas obtained from patients undergoing surgery for hyperparathyroidism in the United States and Western Europe. The adenomas are extracted with 8M urea in 0.2M hydrochloric acid, and fractionated with ether, acetic acid, sodium chloride, and trichloroacetic acid (TCA powder). The TCA powder was further purified by gel and ion exchange chromatography.

Major Findings:

HPTH has been isolated in homogeneous form by the methods outlined above, and detailed in last year's report. During the last year sufficient hormone was isolated to permit the isolation and characterization of the peptide fragments obtained from tryptic digestion of the intact molecule. In addition, procedures were developed for the selective modification of the lysine residues, and subsequent restricted cleavage of the hormone at the arginine residues. These procedures permitted the reinvestigation of the amino terminal sequence of the biologically active region of the hormone which we had previously determined on a relatively small amount of material. An amino acid sequence identical to our previous report was obtained. Thus,

there appears to be no evidence for isohormones for HPTH despite the use of adenomas from over a thousand different patients. During the next year we plan to complete the remainder of the sequence of human parathyroid hormone by automated and manual Edman degradations of the intact hormone, and peptide fragments obtained from tryptic and citroconylated digestions of the native hormones.

2) Objective:

Immunoassay of parathyroid hormone (in collaboration with Dr. Arnaud and colleagues).

Methods Employed:

The methods utilized in these studies have been detailed over the years in the annual reports, and involve labeling of the hormone by the chloramine T method of Hunter and Greenwood, and separation of the antibody-bound and free hormone by dextran-coated charcoal.

Major Findings:

Two groups of antisera were prepared in order to investigate the immunological properties of the synthetic 1-34 peptide (HPTH 1-34) and the intact hormone (1-84). Four antisera were prepared from goats immunized with HPTH (1-34), while the other antisera were obtained from three guinea pigs immunized with crude human PTH extracted from parathyroid adenoma tissue. These antisera were studied by competitive radioimmunoassays under strictly equilibrium conditions. All four goat anti-HPTH (1-34) sera had 2-15 times higher affinity for HPTH (1-34) than for HPTH (1-84), regardless of the labeled antigen (1-34 or 1-84) used. However, the three guinea pig anti-HPTH sera showed 2-12 times higher affinity toward HPTH (1-34) than toward HPTH (1-84) only when HPTH (1-34) was the labeled antigen. With HPTH (1-84) as labeled antigen, HPTH (1-84) exhibited 2-100 times higher affinity than HPTH (1-34). These studies, therefore, indicate that the antigen used for development of the antisera, and the labeled tracer used in the immunoassay significantly influence the type of results obtained in the assessment of the immunological activity of the intact hormone and the amino terminal synthetic peptide. These observations may be explained at least in part by the conformational properties of the two polypeptides (see section 4).

3) Objective:

Determination of the biological activities of HPTH and BPTH, and their respective synthetic amino terminal tetratriacontapeptides (HPTH 1-34, BPTH 1-34) (in collaboration with Dr. Arnaud and colleagues).

Methods Employed:

Adenylate cyclase assays on purified plasma membranes and in vivo rat

bioassays were performed as outlined in previous annual reports.

Major Findings:

Adenylate cyclase assays were performed with purified plasma membrane fractions obtained from rat, chicken and human kidney cortex. The ratio of biological activity utilizing the cyclase assay for the intact bovine and human hormone is as follows: HPTH (1-84):BPTH (1-34); rat 1:25; chicken 1:3; and human 1:1. The synthetic 1-34 human peptide, however, was 15 times more active than the bovine (1-34) peptide in all three systems. The synthetic HPTH (1-34) fragment had 10% of the activity of HPTH (1-84) with the chicken and human cortex system, but had 65% the activity in the membranes isolated from the rat kidney.

In the in vivo rat hypocalcemic bioassay HPTH (1-84) and BPTH (1-34) had almost equal biological activity. However, the BPTH (1-34) fragment was four times more active than the HPTH (1-34) polypeptide.

These results, therefore, indicate that the relative biological potency of HPTH and BPTH and their synthetic fragments are significantly dependent on the assay systems employed. The relative response may reflect differences in molecular structure of the receptors in various species, variation in minimal chain length required to activate the cyclase systems, or differences in degradation-inactivation of the intact hormones and fragments in the various systems studied.

4) Objective:

Comparison of the conformation of HPTH, BPTH, and the synthetic HPTH (1-34) and BPTH (1-34) fragments.

Methods Employed:

Analysis of the secondary or α-helical structure by circular dichroism (CD), and tertiary structure by fluorescence spectroscopy has been detailed in previous annual reports.

Major Findings:

The CD spectra of HPTH, BPTH, HPTH (1-34), and BPTH (1-34) at acidic pH revealed a major trough near 200 nm, which is characteristic of polypeptides in unordered or random conformation. At neutral pH, a significant red shift was observed in the spectra of all four polypeptides. The spectra contained two major troughs at 204 to 208, and at 222 nm, which are the characteristic electronic transitions associated with polypeptides in α-helical conformation. These studies indicate that there is a significant increase in ordered structure in all the polypeptides following titration from acid to physiologic pH.

The fluorescence spectra of the individual hormones and synthetic fragments revealed significant differences. The spectra of HPTH revealed a peak with a maximum at 344 nm, which did not change in 6M guanidine hydrochloride. In HPTH (1-34) the wavelength of tryptophanyl fluorescence was 343 nm, which was normalized to 348 nm in 6M guanidine hydrochloride. In addition, HPTH showed no loss of tryptophanyl fluorescence during titration between pH 8.0 and 11.0 in either aqueous solution or with denaturing reagents. In contrast, HPTH (1-34) demonstrated a 25% loss of tryptophanyl fluorescence in aqueous solution which was normalized in 6M guanidine hydrochloride.

Alkaline titration of BPTH revealed a 20-25% loss of tryptophanyl fluorescence in aqueous solution, which was eliminated in solutions containing denaturing reagents. However, BPTH (1-34) showed no loss of tryptophanyl fluorescence in aqueous solution, and was therefore comparable to the native 1-84 human hormone.

The combined results of these studies indicate these polypeptide hormones and synthetic fragments are not rigid, but can undergo a variety of conformational changes dependent on the aqueous environment. In addition, the conformation of the intact hormones and the synthetic fragments in both HPTH and BPTH appear to be different in the region near the tryptophan residue at position 23. These findings may be of major importance with regard to the immunological cross-reactivity of antibodies prepared against the native hormone or synthetic fragments since conformational differences between the antigens may influence the antibodies produced, and the cross-reactivity of the individual polypeptides. They also provide an explanation for differences in the biological properties of the individual hormones and fragments since degradation or inactivation and receptor site interactions may be profoundly altered by the conformational properties of the constituent polypeptides.

Significance to Biomedical Research and the Program of the Institute:

This work is directed toward a better understanding of the structure, function, and physiological role of polypeptides in cellular metabolism. The determination of the covalent structure of human PTH, and the structure-function determinants of the primary and three-dimensional structure of the hormone will permit the synthesis of selected biologically active and inactive regions of the hormone which may be used for physiological studies, as well as the development of immunological assays for use in the clinical assessment of patients with a variety of disorders of calcium metabolism.

Proposed Course:

During the next year it is planned to complete the covalent structure of human parathyroid hormone. The synthesis of the carboxyl terminal portion of the hormone will then be undertaken by a commercial company in order to develop antibodies against this segment of the structure. It is ultimately planned to develop an immunoassay for the amino as well as the carboxyl

terminal region of the hormone which will then be distributed to the biomedical community for immunoassays in clinical medicine.

Publications:

1. Brewer, H. B., Jr., Fairwell, T., Rittel, W., and Arnaud, C. D.: The chemistry of the parathyroid hormone. Amer. J. Med. 56: 759, 1974.

2. Elson, N. A., Brewer, H. B., and Anderson, W.: Hemoglobin switching in sheep and goats. III. Cell-free initiation of sheep globin synthesis. J. Biol. Chem. 249: 5227, 1974.

3. Arnaud, C. D., Dibella, F. P., Brewer, H. B., Zawistowski, K., and Verheyden, J.: Human parathyroid hormone: biologic and immunologic activities of its synthetic (1-34) tetratriacontrapeptide and the utility of a carboxy-terminal specific radioimmunoassay in assessment of hyperparathyroid syndromes. Proceedings of the Fifth Parathyroid Conference, Excerpta Medica, in press.

4. Brewer, H. B., Jr., Fairwell, T., Ronan, R., Rittel, W., and Arnaud, C.: Human parathyroid hormone. Proceedings of the Fifth Parathyroid Conference, Excerpta Medica, in press.

5. Arnaud, C. D., and Brewer, H. B., Jr.: Parathyroid hormone: structure and immunoheterogeneity. In Simmons, I. L., and Ewing, G. W. (Eds.): Methods in Radioimmunoassay, Toxicology, and Related Areas, New York, Plenum Publishing Corp., 1974, p. 45.

Key Words:

1) peptide
2) parathyroid hormone
3) immunoassay
4) circular dichroism
5) biological activity
6) adenyl cyclase
7) amino acid sequence
8) conformation
9) fluorescence
10) tertiary structure

Project No. Z01 HL 02010-05 MDB
1. Molecular Disease Branch
2. Section on Peptide Chemistry
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Structure and Function of the Plasma Lipoproteins

Previous Serial Number: None

Principal Investigators: H. Bryan Brewer, Jr., M.D.
Gerd Assmann, M.D.

Other Investigators: Ann LaRue, B.A.
Anne Flory, B.S.

Cooperating Units: Harold Edelhoch, Ph.D., John Gwynne, M.D., and James Osborne, Ph.D., Clinical Endocrinology Branch, NIAMDD
Edward Sokoloski, B.S. and Robert Highet, Ph.D., Chemistry Branch, NHLI

Project Description:

1) Objective:

Determination of the molecular organization of the phospholipids in native high density lipoprotein (HDL).

Methods Employed:

The spatial orientation of the phospholipids of HDL was assessed by phosphorus nuclear magnetic resonance ($^{31}$P NMR) utilizing Fourier transform technique.

Major Findings:

VLDL, LDL, and HDL all exhibited characteristic $^{31}$P NMR spectra containing two major resonances. These two resonances were separated by 0.6 ppm and could be assigned to phosphatidylcholine and sphingomyelin. The relative intensities of the NMR spectra of the two phospholipids agreed very well with chemical determinations of the individual lipids. In order to evaluate the solvent accessibility of the polar headgroups of the phospholipids, native HDL and synthetic phospholipids were titrated with the paramagnetic ion, europrium. When phospholipids in bilayered structure are titrated with paramagnetic ions, the electronic transitions of the polar headgroups that are exposed to the aqueous solvent are shifted upfield. The inner phosphorus

groups are shielded and remain uncomplexed with the titrating reagent. Phospholipids in micellar structure have all the polar headgroup exposed. Titration of native HDL revealed complete broadening and shifting of the phosphorus peaks suggesting that all of the phospholipid polar headgroups were available for binding. These studies suggest that the lipid in HDL is organized into a spherical micelle, rather than in a classical membrane bilayer. These results are consistent with recent low angle x-ray diffraction studies which indicate that HDL has an organized symmetrical structure with a relatively electron-poor (nonpolar) central region, and a relatively electron-rich (polar) outer region (Shipley, et al., J. Supramolecular Struc. 1, 18, 1972).

2) Objective:

Lipid-protein interactions of A-I and A-II with the phospholipids, phosphatidylcholine (PC) and sphingomyelin (SPM).

Methods Employed:

The apolipoproteins were recombined with phospholipid in aqueous solution without sonication. The recombined particles were isolated by gel filtration or preparative ultracentrifugation. The isolated complex was evaluated for the molar ratio of lipid to protein, and by circular dichroism for changes in conformation concomitant with lipid-protein binding.

Major Findings:

ApoHDL, apoA-II, and reduced and carboxymethylated apoA-II readily recombined with PC or SPM to form particles that were similar in size to native HDL. The carboxyl terminal, but not the amino terminal cyanogen bromide fragment of A-II, recombined with lipid. ApoA-I, and the carboxyl terminal cyanogen bromide fragment of A-I, however, recombined with PC or SPM to only a very limited extent. A-I in the presence of A-II, however, readily recombined. These studies suggest that A-II may influence the structure of A-I, permitting lipid-lipid interaction, or that A-I may be held in the recombined particle by protein-protein interactions.

Analysis of the recombined particles by circular dichroism indicated that there was an increase in helical structure concomitant with lipid-protein interaction.

3) Objective:

Determination of the quaternary structure of native HDL.

Methods Employed:

The quaternary structure of HDL has been evaluated by a variety of techniques including $^{13}C$ NMR, CD, molecular modeling, and recombination of the constituent apoproteins with isolated phospholipids.

Major Findings:

A variety of approaches have been employed in order to gain some insight into the molecular organization of lipids and proteins in HDL. The major force involved in lipid-protein interactions has been evaluated by $^{13}C$ NMR of PC and SPM specifically enriched in the polar headgroups. Spin lattice relaxation times of recombined apoproteins, and model compounds were identical, indicating that the polar headgroups of PC and SPM were in the same hydrophilic environment in either sonicated lipid particles or reassembled lipoproteins. These studies suggest that ionic binding is of minor importance in lipid-protein interactions. The major force, therefore, is hydrophobic in nature. A significant increase in helical structure has been observed following the recombination of apolipoproteins with lipids. Models of apolipoproteins in α-helical conformation have demonstrated the presence of amphipathic surface areas. One surface is hydrophilic, while the other surface is hydrophobic in nature.

The combined results from these and other studies have permitted the development of a molecular model for HDL. In this model HDL is a spherical micelle with the globular proteins visualized as "icebergs" in a "sea" of lipid. The major force involved in lipid-protein interaction is hydrophobic in nature. The specific orientation of the A-I and A-II in the particle is as yet unknown. Recombination studies outlined above would suggest, however, that protein-protein interactions between A-I and A-II may be of importance in the molecular organization of HDL. This model is quite different than the "classic" model of lipoproteins in which the lipid portion of the particle was conceptualized as an "oil droplet" with the protein covering the surface of the droplet. The spatial organization of the neutral lipids, cholesteryl ester and triglyceride are as yet unknown. However, x-ray diffraction data would suggest that they are "core" constituents of the lipoprotein particle.

4) Objective:

Determination of the molecular properties of A-I in aqueous solution and in native HDL.

Methods Employed:

The molecular properties of A-I have been evaluated by fluorescence, difference absorption spectroscopy, and circular dichroism.

Major Findings:

Native A-I has a high degree of helical content in aqueous solution at neutral pH. This helical content is partially eliminated in acid, more extensively lost in alkali, and almost destroyed in 1.7M guanidine hydrochloride. The fluorescence behavior of A-I revealed a high degree of ordered structure with the major fluorescence peak near 333, indicating that the

tryptophanyl residues are shielded from the aqueous environment. Denaturation of A-I with 1.7M guanidine hydrochloride caused a normalization of the fluorescence spectra. Thus, A-I behaves as a typical globular protein with a high degree of ordered structure.

The unique absence of tryptophanyl residues in A-II has permitted the direct analysis of the molecular behavior of A-I (contains 4 tryptophan residues) in native HDL. Analysis of A-I by the procedures outlined above has demonstrated a marked increase in stability of A-I in HDL to changes in temperature, pH and guanidine hydrochloride. Therefore, the tertiary structure of A-I is markedly stabilized when incorporated into native HDL. The microenvironment of A-I in HDL which imparts the unique stability to the intact lipoprotein particle is as yet unknown. These forces may involve polar phospholipids, non-polar lipids or other apolipoproteins. Further studies will be required to clarify the specificity of the interaction of A-I with lipids, and with other apolipoproteins.

5) Objective:

Determination of the molecular properties of A-II from HDL.

Methods Employed:

The evaluation of the molecular properties of A-II was performed by the procedures outlined above.

Major Findings:

A variety of physical studies have clearly indicated that A-II, similar to A-I, has a high degree of ordered structure in aqueous solution. The molecule, however, is not a rigid structure, and undergoes extensive loss of structure with extremes of pH and low concentrations of guanidine hydrochloride. A unique feature of A-II, however, was discovered which indicated that A-II self-associates under various protein concentrations. In the concentration range investigated, A-II self-associates to an oligomeric species which is consistent with a monomer-dimer equilibrium. The self-association of A-II is associated with significant increases in secondary and tertiary structure. This reversible association was found to be dependent on temperatures between 5 and 50°C, showing a maximum in association near 25°C. The observation that A-II self-associates is undoubtedly of major importance in the molecular organization of A-II in HDL, and may indicate that a specific oligomeric species may be the active molecular form which will recombine with lipids.

6) Objective:

Evaluation of the binding of A-I to lysolecithin.

Methods Employed:

Similar to those outlined above.

Major Findings:

The molecular binding of A-I to lysolecithin was investigated for two reasons: first - A-I is known to be a cofactor for the enzyme lecithin cholesterol acyl transferase (LCAT) which generates lysolecithin following the transfer of the fatty acid side chain of lecithin to the hydroxyl group of cholesterol; second - A-I is unable to bind PC or SPM to any significant degree without the presence of A-II in the recombination mixture. A-I in these studies was able to bind lysolecithin with an association constant greater than $10^7$. The binding appears to be to the lysolecithin monomer, with ultimate formation of a lipid-protein complex that could be isolated by gel permeation chromatography. Of major interest was the ability of lysolecithin to bind to A-I in the presence of 1.8M guanidine hydrochloride. A 25 amino acid cyanogen bromide fragment of A-I also binds lysolecithin as strongly as the native molecule. These studies would, therefore, suggest that the binding of A-I to lysolecithin may be related to the primary structure of the protein.

Significance to Biomedical Research and the Program of the Institute:

This work is directed toward a general understanding of the molecular forces involved in lipid-protein interactions, with the ultimate goal of understanding the molecular architecture of the plasma lipoproteins. It is assumed that a fundamental understanding of the quarternary structure of the plasma lipoproteins will permit more sophisticated studies to be performed on the functions of the circulating lipoproteins. The mode of lipid transport of these lipoproteins, the metabolism, and interconversions of the various lipoprotein families will be of fundamental importance in the 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 it is planned to continue a detailed examination of the molecular properties of the individual apoproteins, and to initiate studies on the interactions between the apoprotein both in aqueous solution and in recombined particles. Systematic studies will also be continued on the nature of the lipid organization in recombined lipoprotein particles. The molecular model for HDL presented in this report will be tested by a variety of different approaches including isosteric modifications of the apolipoprotein as well as structural perturbations of the lipid structure. It is hoped in the near future to be able to begin synthetic studies on the apolipoproteins which would permit more detailed studies on the nature of lipid-protein interactions.

As in the past, we plan to continue our studies on the isolation and

characterization of the human apolipoproteins, as well as the apolipoproteins from a variety of animal species. In addition, it is planned to complete the amino acid sequence of human A-I. Detailed analysis of the different polymorphic forms of A-I is nearly complete. Theses studies will permit more detailed analysis of the role of polymorphism in the structure and immunological properties of the plasma lipoproteins.

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Key Words:

1) lipid
2) sequence
3) conformation
4) NMR (nuclear magnetic resonance)
5) lipoprotein
6) self-association
7) lysolecithin
8) lecithin

Annual Report of the
Molecular Hematology Branch
National Heart and Lung Institute
July 1, 1974 through June 30, 1975

The factors influencing the regulation of hemoglobin gene expression are being studied in human and animal erythroid cells. The objective is to understand the molecular basis of hereditary anemias (specifically the thalassemias and hemoglobinopathies) in order to be able to devise effective methods for treating these diseases. The general approach has been to fractionate normal and diseased erythroid cells into the various components required for hemoglobin synthesis and then to reconstitute the cellular components in such a way as to reproduce the activity of the original intact cell. The function of each cellular component from the diseased cell can then be compared with its counterpart from a normal cell. The first phase of this program has been successfully completed, namely, the fractionation of the cytoplasm of normal and diseased reticulocytes and the reconstitution of a cell-free system capable of duplicating the normal and disturbed functions of reticulocyte cytoplasm. A detailed characterization of purified cytoplasmic components (initiation factors, elongation factors, ribosomes, messenger RNA, etc) is being carried out in order to understand fully the mechanism of protein synthesis in mammalian cells.

Fractionation of the nucleus of erythroid precursors is the second phase of the program. During the past year the cell-free system which is capable of transcribing globin messenger RNA sequences from bone marrow chromatin has been refined. Although this assay is still relatively crude, it should allow a search for the factors involved in the regulation of the synthesis of specific globin messenger RNA.

The Molecular Hematology Branch has as its goal the treatment of human genetic diseases, specifically, thalassemia and sickle cell anemia. Several approaches have been initiated: First, as described above, is the complete fractionation of the erythroblast so as to allow a study of the factors involved in messenger RNA and protein synthesis. With this knowledge ways will be sought for influencing globin messenger RNA production in thalassemic cells. Second, the mechanism of globin gene switching is under active investigaton using the animal model system of the hemoglobin A to hemoglobin C gene switch in sheep and goats. The goal is to learn the mechanism of this switch in order to understand how to obtain adult to fetal gene switching in patients who have normal fetal but abnormal adult hemoglobin. Third, somatic cell hybrids generated by fusion of erythroid cells and established cell lines have been used in an attempt to establish replicating cells capable of globin synthesis. These hybrids are being utilized to study regulation of expression of the globin genes. In addition, human-mouse hybrids containing one or more human chromosomes are being studied in order to determine the localization of the human alpha and beta globin structural genes.

The Clinical Service of the Molecular Hematology Branch, opened two years ago, has greatly expanded. Patients with various blood cell disorders including thalassemia, sickle cell anemia and other hemolytic anemias are

diagnosed, treated and studied. Several clinical protocols are under investigation.

A summary of our results obtained over the past twelve months for projects continued from past years follows:

1) Initiation factors: An intensive effort continues to be made to identify, purify, and characterize the eukaryotic initiation and elongation factors. Of the seven known initiation factors, five have been purified to homogeneity and have been fully characterized. An intensive effort is now under way to locate possible messenger RNA specific initiation factors which might play a role in translational control of protein synthesis.

2) Mechanism of hemoglobin initiation: As the various initiation and elongation factors have been purified they have been utilized to determine the exact step-by-step mechanism of hemoglobin synthesis. The overall picture of initiation of eukaryotic protein synthesis is now reasonably well established. The essential role of initiation factor MP has been elucidated.

3) Molecular basis of thalassemia: Patients with various types of thalassemia have been studied and shown to have a quantitative decrease in the amount of messenger RNA for the affected globin chain. Messenger RNA from these patients have been utilized to prepare human globin chain complementary DNA for several studies (see section 6 and 7, below).

4) Hemoglobin A → C switch in sheep and goats: Sheep and goat bone marrow cells capable of generating erythroid colonies in tissue culture have been separated by unit gravity sedimentation. The population of colony forming cells responsible for the A → C switch appears to have been identified. Utilizing the experience gained in tissue culture of sheep and goat erythroid cells, human erythroid colonies have been grown from bone marrow of patients with various hemolytic anemias. The concepts and techniques developed for study of A → C switch in cells of sheep and goats are being applied to the study of regulation of human hemoglobin synthesis.

5) Synthesis of globin DNA gene: The mechanism of action of the viral enzyme, RNA-directed DNA polymerase, is under investigation. Using short single-base oligonucleotides of defined length it has been shown that the enzyme transcribes various ribonucleotide bases in different ways: slippage with oligo(rA), faithful transcription with oligo(rC), and poor transcription with oligo(rU) and oligo(rG). This variability in enzyme behavior may account for the fact that the enzyme does not transcribe natural messenger RNA templates completely.

6) Cell-free synthesis of globin messenger RNA: A hybridization assay has been devised which reproducibly quantitates globin messenger RNA sequences transcribed in vitro from bone marrow chromatin using the enzyme RNA polymerase. This assay system should permit the systematic investigation of the variables affecting specificity of in vitro transcription. Chromatin from human bone marrow can now be utilized for studies to quantitate the synthesis of alpha and beta globin messenger RNA sequences.

7) Globin gene expression in somatic cell hybrids: Hybrid cells have been obtained from the fusion of human marrow cells with Friend mouse erythroleukemia cells which exhibit either: co-expression of human and mouse globin,

expression of mouse globin only, or total absence of globin gene expression. In one somatic cell hybrid, human beta globin messenger RNA but no human alpha globin messenger RNA was detected. These data indicate that the alpha and beta globin structural loci are on separate chromosomes. In addition, somatic hybrid cells have been generated which contain a complete complement of rodent chromosomes but only one or more human chromosomes. These hybrids are being utilized with the technique of complementary DNA-DNA hybridization to localize the human alpha and beta structural globin genes.

8) Clinical investigations. (a) Iron chelation therapy in transfusional hemosiderosis is being evaluated using the agent Desferal administered either with or without ascorbic acid. It has been shown that iron-loaded patients can be placed in negative iron balance with Desferal and that this can, under some circumstances, be markedly enhanced by the simultaneous administration of ascorbic acid. (b) Cardiac hemolytic anemia. The degree of anemia has been shown to have no consistent effect on the hemolytic rate in patients with cardiac hemolysis. In addition, several patients have been shown to have mild or moderate hemolysis but severe anemia; this reflects inadequate bone marrow compensation.

A report on the new programs established during the past 12 months follows:

1) Regulation of the respiratory function of blood. A program has been established which is aimed at understanding the mechanisms which regulate the respiratory function of blood at molecular, cellular and physiologic levels. Structure-function relationships are studied in normal and mutant human hemoglobins. A new hemoglobin variant has been discovered which appears to be the first human protein whose structural alteration has arisen by mutation which corresponds to a "nonsense" mutation in bacteria. Two amino acids are lacking at the mutant's carboxyl terminal end. This hemoglobin has the highest oxygen affinity of any known human variant, nonetheless carriers of it are asymptomatic.

2) Evaluation of alteration of blood oxygen affinity as the basis for the treatment of sickle cell anemia. This recently established project is designed to study the relationship between sickling and the oxygen transport of blood. Patients with sickle cell anemia are admitted to the Clinical Center and studied intensively using a number of biochemical and physiologic techniques.

Project No. Z01 HL 02201-03 MHB
1. Molecular Hematology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Mechanism of hemoglobin biosynthesis in cell-free systems

Previous Serial Number: NHLI-88

Principal Investigator: William C. Merrick, Ph.D.

Other Investigators: Brian Safer, M.D., Ph.D.
Sherrill Adams, Ph.D.
Wayne Kemper, M.A.
Irving London, Ph.D.
W. French Anderson, M.D.

Cooperating Units: Molecular Disease Branch, NHLI
Laboratory of Nutrition and Endocrinology, NIAMDD
Development Immunology Branch, NICHHD
Roche Institute of Molecular Biology
Massachusetts Institute of Technology

Project Description:

Objectives: The mechanism and regulation of the initiation of mammalian protein synthesis is being investigated by utilizing artificial template or hemoglobin mRNA-directed protein biosynthesis. Components from the rabbit reticulocyte have been fractionated and highly purified. Reactions of either the separate components or combinations of components are being studied to elucidate the sequential steps involved in protein synthesis.

Methods: Purification of separate components has been achieved using standard techniques such as ion-exchange column chromatography, gel filtration, salt fractionation, and sucrose density gradient centrifugation. Physical characterization of initiation and elongation factors (IF and EF, respectively) have been performed using gel electrophoresis in various buffers, gel filtration, analytical centrifugation, and amino acid analysis. Biological characterization of components of protein biosynthesis has been developed or modified from techniques described in the literature.

Major Findings: 1) IF-M2A has been purified to homogeneity and characterized. IF-M2A is active as a single polypeptide chain of molecular weight 125,000. The amino acid composition is unusual in that glutamic acid (plus glutamine) constitutes 18.8 mole percent while cysteine and tryptophan consititute only 0.7 and 0.4 mole percent, respectively. IF-M2A has a frictional ratio of 1.66 and a pI of 6.45. The expression of GTPase activity by IF-M2A has been shown to require both 40S and 60S subunits.

2) IF-M2Bα has been purified to homogeneity. IF-M2Bα self aggregates at protein concentrations greater than 0.1 mg/ml and consequently limited physical studies have been possible. IF-M2Bα has molecular weight of 16,500 and is active as a single polypeptide chain. Although the precise role for either IF-M2Bα or IF-M2Bβ in protein synthesis is still unclear, these proteins appear to be involved mostly with subunit joining and not with binding of the initiator tRNA.

3) IF-MP has been purified to homogeneity. This initiation factor is different from the others which have been purified in this laboratory in that it is the only one composed of subunits (35,000 and 55,000 daltons). Physical studies indicate that IF-MP may exist as a dimer (90,000 daltons) or a tetramer (180,000 daltons); however, the biologically active form has not been determined. IF-MP forms a ternary complex with GTP and initiator tRNA which can be subsequently bound to 40S subunits.

4) A comparison of the initiation factors which have been physically characterized indicate several common features: 1) IF-M1, IF-M2A, and IF-MP all have frictional ratios greater than 1.5; 2) the ratio of lys:his:arg is approximately 10:2:5 with the total basic amino acid content being 15 to 20 mole percent; 3) all of these factors are inactivated by N-ethylmaleimide.

5) A complex of initiator tRNA and 40S subunits can be formed either by IF-MP or IF-M1. This 40S complex subsequently requires IF-M2A, IF-M2B, 60S subunits and GTP to generate an 80S initiation complex which can react with puromycin (however, the template AUG must also be present during the formation of the 40S or 80S complex). Similar studies utilizing salt-washed ribosomes with endogenous mRNA indicate that IF-MP, but not IF-M1, is capable of generating a puromycin sensitive 80S complex.

6) IF-MP has been shown to be able to relieve the inhibition of globin synthesis in crude lysate systems which contain either double-stranded RNA or oxidized glutathione or which are hemin-deficient. The inhibition of globin synthesis does not appear to be a direct effect on IF-MP, but rather an indirect effect mediated by a protein which is present only at times when globin synthesis is inhibited. IF-MP is also capable of selectively binding globin mRNA and poly(A) (in addition to Met-$tRNA_f$). This protein has also been isolated from mRNP particles suggesting that IF-MP may play a role in both mRNA and Met-$tRNA_f$ recognition in the formation of an initiation complex.

7) Treatment of rabbit reticulocyte EF-1 with either phospholipase C or phospholipase AB causes a change in the molecular weight of EF-1 from 800,000 to 200,000 without loss of biological activity. This observation supports the suggestion that rabbit reticulocyte EF-1 (like EF-1 from other tissues) is composed of subunits (in this case 47,000 and 50,000 daltons) which are held together to some extent by phospholipids.

Significance to Biomedical Research and Institute Program

An understanding of the regulation of gene expression is essential to evaluate normal and abnormal cell function. The observation that IF-MP may play some regulatory role in globin biosynthesis lends support to the idea that regulation of the initiation of protein synthesis may be an important part of globin gene expression. Similar mechanisms may also be operative in other tissues.



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Proposed Course of Project: We plan to continue purification and characterization of those factors which have not been fully evaluated. With the homogeneous factors available and those to be obtained, studies with exogenous mRNA are planned to explore fully the differences in factor requirements for protein synthesis initiation with natural mRNA as opposed to artificial templates. In addition, studies are planned to examine the differences between mRNA and mRNP particles as well as factors which might effect the ratio of α and β globin chain synthesis.

Keyword Descriptors: reticulocyte, initiation factors, elongation factors, protein biosynthesis, protein purification, molecular weight, methionyl-puromycin, hemin regulation, mRNA, poly(A).

Honors and Awards: None

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Project No. Z01 HL 02202-03 MHB
1. Molecular Hematology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Evolution of the Protein Synthesizing Machinery

Previous Serial Number: NHLI-89

Principal Investigator: William C. Merrick, Ph.D.

Other Investigators: Norton Elson, M.D.
Brian Safer, M.D., Ph.D.
Sherrill Adams, Ph.D.
Severo Ochoa, Ph.D.
W. French Anderson, M.D.

Cooperating Units: Roche Institute Molecular Biology

Project Description:

Objectives: To evaluate the interchangeability of components from various organisms which are required for protein biosynthesis and thus determine the level of evolutionary homology in both the components and mechanisms of protein biosynthesis.

Methods: Protein and nucleic acid components from different sources are isolated and compared in cell-free assay systems.

Major Findings: 1) A comparison has been made of the ability of the formylated and unformylated initiator tRNAs of E. coli and rabbit liver to participate in a number of model reactions of protein synthesis. The results indicate that the prokaryotic initiator tRNA species functions efficiently in partial reactions which involve only binding, but that the methionine donated by the prokaryotic tRNA is not incorporated efficiently into peptide linkage. The effect of formylation on the effectiveness of the initiator tRNA is not clear; it reduces activity in ternary complex formation, does not affect AUG-directed binding to 40S subunits, and increases the rate or extent of incorporation of methionine, or both, into methionyl-puromycin and globin chains.

2) In the reticulocyte lysate, AUG-directed methionyl-puromycin synthesis requires methionyl-tRNA$_f$, 40S and 60S subunits, IF-M1, IF-M2A, IF-M2B and GTP. In the Artemia salina (brine shrimp) system, AUG-directed methionyl-puromycin synthesis requires only methionyl-tRNA$_f$, 40S and 60 S subunits, and EIF-1 (an initiation factor corresponding to IF-M1). In crosses between these two systems, it has been shown that the requirement for IF-M2A, IF-M2B and GTP is due to the differences between rabbit and Artemia salina 60S subunits. When rabbit reticulocyte 60S subunits are used with either rabbit or Artemia salina 40S



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subunits (with either IF-M1 or EIF-1), IF-M2A, IF-M2B and GTP are required. IF-M2A, IF-M2B, and GTP are not required if Artemia salina 60S subunits are used. These results suggest that Artemia salina 60S subunits contain proteins corresponding to IF-M2A and IF-M2B.

Significance to Biomedical Research and Institute Program: The ability of prokaryotic components to interact in eukaryotic protein synthesis details both the ways in which the process is similar as well as the manner in which they are distinct. The ability to distinguish between bacterial and mammalian cell functions is a large part of the basis for the formulation of therapeutic treatment of bacterial infection. The continued study of such differences as well as differences between eukaryotic organisms may lead to new and unique treatments for human diseases (bacterial, viral or parasitic).

Proposed Course of Project: The species specificity of the cell component required for protein biosynthesis will continue to be investigated using both artificial and natural mRNA templates.

Keyword Descriptors: evolution, E. coli, rabbit reticulocyte, Met-$tRNA_f$, f Met-$tRNA_f$, initiation complex, globin biosynthesis, methionyl-puromycin, initiation factors.

Honors and Awards: None

Publications:

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Project No. Z01 HL 02205-02 MHB
1. Molecular Hematology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Mechanism of Action of the Enzyme RNA-Directed DNA Polymerase

Previous Serial Number: NHLI-92

Principal Investigator: Gary Weiss, M.D., Ph.D.

Other Investigators: Judith Kantor, Ph.D.
Amy Falvey, Ph.D.
W. French Anderson, M.D.

Cooperating Units: Viral Oncology Branch, NCI

Project Description:

Objectives: The enzyme RNA-directed DNA polymerase, also known as Reverse Transcriptase, from avian myeloblastosis virus (and other sources) provides a possible means for synthesizing a DNA gene directly from isolated messenger RNA. Unfortunately, the DNA product so far obtained is only a partial copy of the messenger RNA template. It is desired, therefore, to learn the mechanism of action of the enzyme in order to establish conditions whereby a complete (and active) DNA gene can be transcribed from a globin messenger RNA.

Methods: The enzyme is purified by standard procedures from avian myeloblastosis virus obtained through the National Cancer Institute. Globin messenger RNA is isolated from rabbit reticulocytes; DNA product analysis is by $CeSO_4$ density gradient centrifugation. Primers and single base oligonucleotides are prepared by enzymatic and/or chemical means followed by purification by standard nucleic acid fractionation techniques. Artificial block polynucleotide templates are being synthesized by means of various enzymatic and chemical techniques including use of the enzyme polynucleotide phosphorylase.

Major Findings: 1) The synthesis of DNA products complementary to a range of artificial templates by the enzyme RNA-directed DNA polymerase has been studied. Of the single base ribopolynucleotides, poly(C), poly(A), and poly(I) were active while poly(G) and poly(U) were almost inactive. Of the deoxypolynucleotides tested, only poly(dC) showed significant activity.

2) In order to examine the fidelity of transcription, single base oligonucleotides of defined length were studied. The minimum length showing activity for an oligo(rC) template was 9; the minimum primer length of oligo(dG) was 3 or 4. Using $(rC)_{13}$ as template and $(dG)_8$ as primer, the oligo(dG) product coelectrophoresed with the template. However, using $(rA)_{20}$ as template and $(dT)_{10}$ as primer, a very large (10-16S) product was formed. No significant activity was obtained with oligo(rU) templates. It therefore appears that RNA-directed DNA polymerase transcribes the various ribonucleotide bases in different ways: slippage with oligo(rA), faithful transcription with oligo(rC), and poor

transcription with oligo(rU).

3). The Km has been determined for each substrate. By increasing the concentration of nucleotide triphosphate in the reaction mixture a higher percentage of large (10S) cDNA can be obtained. Studies to determine if this 10S cDNA is a faithful copy of the mRNA template are underway.

Significance to Biomedical Research and Institute Program: The ability to synthesize a DNA gene in vitro would be a major step towards the goal of successful therapy of human genetic diseases.

Proposed Course of the Project: Detailed studies on the mechanism of action of the enzyme RNA-directed DNA polymerase are being carried out.

Keyword Descriptors: Reverse transcriptase, complementary DNA, messenger RNA, enzyme kinetics, oligonucleotides

Honors and Awards: None

Publications:

1. Falvey, Amy K., Kantor, Judy A., Robert, M.G., Picciano, Dante J., Weiss, Gary B., Vavich, Joel M., and Anderson, W. French: Mechanism of action of RNA-directed DNA polymerase. I. Transcription of globin messenger RNA. J. Biol. Chem. 249: 7049-7056, 1974.

Project No. Z01 HL 02206-02 MHB
1. Molecular Hematology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Mechanism of Globin Messenger RNA Transcription in Bone Marrow Cells

Previous Serial Number: NHLI-107

Principal Investigators: Alan W. Steggles, Ph.D.
Golder Wilson, M.D., Ph.D.

Other Investigators: Judith Kantor, Ph.D.
Arthur Nienhuis, M.D.
W. French Anderson, M.D.
Joseph E. Pierce, D.V.M.
Leonard Stuart

Cooperating Units: Section on Laboratory Animal Medicine and Surgery, NIH
Ungulate Unit, NIH Animal Center

Project Description:

Objectives: The objective of this project is to determine the normal mechanism of regulation of globin messenger RNA synthesis in bone marrow cells. Specific regulatory factors, either protein or RNA molecules, will be sought which influence the transcription of the individual human or sheep globin genes. These studies will be applied to chromatin from cells of patients with beta thalassemia to provide further insight into molecular defect in this disease.

Methods: Young sheep are the source of bone marrow and other tissues. Chromatin and chromatin fractions (histones, acidic proteins, DNA) are obtained by standard biochemical techniques. DNA-dependent RNA polymerase is purified from sheep liver. Messenger RNA is detected by hybridization with globin complementary DNA. Detection of the hybrid is by analysis with single strand specific nuclease. Alternatively, after purification of the cDNA·RNA hybrid by preliminary ribonuclease digestion and hydroxylapatite chromatography, it is detected by cesium sulfate buoyant density centrifugation. The chromosomal proteins are dissociated from DNA by high salt in concentrated urea and the non-histone protein fraction purified by ion exchange chromatography. These non-histone proteins are reconstituted to liver chromatin.

Major Findings: 1) Globin gene transcription by bacterial polymerase has been definitively confirmed by demonstration of labeled RNA sequences which are synthesized *in vitro* and hybridize to globin cDNA.

2) A hybridization assay has been devised which reproducibly quantitates globin sequences permitting systematic investigation of the variables affecting the specificity of *in vitro* transcription.

3) Initial evidence suggests that RNA transcription in vivo is asymmetrical (only one DNA strand is transcribed) but that the in vitro reaction with bacterial polymerase appears to be symmetrical in that there is transcription of both the globin gene and the DNA strand complementary to it.

Significance to Biomedical Research and Institute Program: Understanding the mechanism of messenger RNA transcription in eucaryotic cells is essential to understanding gene action.

Proposed Course of the Project: Reconstitution of bone marrow non-histone proteins to non-erythroid chromatin may provide a means to assay specific non-histone protein fractions for regulatory factors. Purification of the globin genes by fractionation of chromatin will be pursued in an effort to obtain DNA fragments also containing sequences of nucleotides which may interact with regulatory proteins.

Keyword Descriptors: Gene regulation, histones, non-histone chromosomal proteins, chromatin, nucleic acid hybridization, RNA polymerase, messenger RNA synthesis, globin genes erythroid cells.

Honors and Awards: None

Publications:

1. Anderson, W.F., Steggles, A., Wilson, G., Kantor, J., Velez, R., Picciano, D., Falvey, A., and Nienhuis, A.: Cell-free transcription of human bone marrow chromatin. Ann. N.Y. Acad. Sci. 241:566-581, 1974.

2. Anderson, W.F., Barker, J.E., Elson, N.A., Merrick, W.C., Steggles, A.W., Wilson, G.N., Kantor, J.A., and Nienhuis, A.W.: Activation and inactivation of genes determining hemoglobin phenotypes. J. Cell Physiol. 85:477-494, 1975.

Project No. Z01 HL 02207-02 MHB

1. Molecular Hematology Branch
2.
3. Bethesda, Maryland 20014

PHS-NHLI
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Globin Gene Expression in Somatic Cell Hybrids

Previous Serial Number: NHLI-94

Principal Investigators: Albert Deisseroth, M.D., Ph.D.
Arthur W. Nienhuis, M.D.

Other Investigators: Ramon Velez, M.D.
Jane Barker, Ph.D.
W. French Anderson, M.D.

Cooperating Units: Laboratory of Biochemical Genetics, NHLI
Department of Biology, Yale University

Project Description:

Objectives: Somatic cell hybrids, generated by fusion of erythroid cells and established cell lines, will be used to obtain continuously replicating cells capable of globin synthesis. These would then be utilized to study regulation of expression of the globin genes. In addition, human-mouse hybrids containing one or more human chromosomes would be studied for localization of human globin structural genes.

Methods: Erythroid cells are obtained from human and animal bone marrow, mouse spleen, or fetal liver and purified by differential immune lysis, buoyant density centrifugation, or unit gravity sedimentation. These cells are fused to established cell lines using inactivated Sendai virus. Selective media are constructed which favor the growth of the hybrid cells over the parent lines and the hybrid nature of the resulting clones is confirmed by analysis of the chromosomal composition and isozyme content. Globin messenger RNA is detected by hybridization of the hybrid cell RNA to appropriate complementary DNA probes and the presence of globin is detected by radioactive precursor incorporation. Gene localization studies require DNA-cDNA hybridization using fractionated and species specific globin cDNA probes.

Major Findings: 1) Hybrids were obtained from fusion of human marrow cells with Friend mouse erythroleukemia cells which exhibit either: co-expression of human and mouse globin, expression of mouse globin only, or total absence of globin gene expression. Two of the human X mouse hybrid cell lines exhibited DMSO induction of both human and mouse globin synthesis as determined by annealing of the cellular RNA to the specific mouse and human globin complementary DNAs (cDNA). Alpha enriched and beta enriched human cDNAs were prepared using beta thalassemia and HbH disease reticulocyte mRNAs, respectively. One of the two positive human X mouse hybrids contained human beta mRNA but not alpha. Purified hemoglobin labeled by incubation of these hybrid cells with [$^{3}$H]leucine contained [$^{3}$H] beta globin and mouse globins, but no human alpha globin.

These data indicate that the alpha and beta structural loci are on separate chromosomes.

2) The pattern of globin gene expression was correlated with the chromosomal composition of the hybrid cells by karotyping, Giemsa banding and isozyme analysis. Loss of the human biarmed chromosomes present in cell hybrids exhibiting co-expression of human and mouse globin genes resulted in loss of human but not mouse globin mRNA synthesis.

3) In order to identify the specific chromosomes bearing human globin genes, homogeneous stable populations of Chinese hamster X human cell hybrids containing one human chromosome and a full complement of Chinese hamster chromosomes were generated. In the several hybrid cells studied thus far, human globin genes were not detected by DNA-cDNA hybridization.

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 opportunity for insight into genetic regulation by exploiting our knowledge of and ability to study the expression of the globin genes in stable somatic cell hybrids.

Proposed Course of the Project: The studies leading to the chromosome assignment of the alpha and beta globin structural genes will be continued. Somatic cell hybrids which coexpress both mouse and human globin chains will be studied to attempt to learn the factors controlling expression of the globin genes.

Keyword Descriptors: Erythroleukemia, Friend cell, virus, molecular hybridization, complementary DNA, messenger RNA, gene mapping, erythroid cells, human bone marrow, Chinese hamster.

Honors and Awards: None

Publications:

1. Deisseroth, A., Burk, R., Picciano, D., Minna, J., Anderson, W.F., and Nienhuis, A.W.: Expression of globin genes in somatic cell hybrids: I. Synthesis in human marrow-mouse erythroleukemia hybrid cells. Proc. Nat. Acad. Sci. USA, 72:1102-1106, 1975.

2. Deisseroth, A., Barker, J., Anderson, W.F., and Nienhuis, A.: Hemoglobin synthesis in somatic cell hybrids: II. Coexpression of mouse with human or Chinese hamster globin genes in interspecific somatic cell hybrids of mouse erythroleukemia cells. Proc. Nat. Acad. Sci. (in press).

Project No. Z01 HL 02210-01 MHB
1. Molecular Hematology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Mechanisms of Regulation of the Respiratory Function of Blood

Previous Serial Number: None

Principal Investigator: Robert M. Winslow, M.D.

Other Investigators: Mei-Lie Swenberg, Ph.D.
R. L. Berger, Ph.D.
Ehrhard Gross, Ph.D.

Cooperating Units: Laboratory of Technical Development, NHLI
Section of Molecular Structure, Reproduction
Research Branch, NICHD

Project Description:

Objectives: The objective of this project is the understanding of the mechanisms which regulate the respiratory function of blood at molecular, cellular, and physiologic levels. The oxygenation of normal human hemoglobin, variant human hemoglobins, and hemoglobins of animals are studied, along with the cellular factors which modify these properties. The ultimate objective of the project is to understand the relationships among these properties, pulmonary gas exchange and tissue oxygen demands, under normal and pathologic conditions.

Methods: 1) A continuous automatic oxygenation apparatus based on a Cary 118C Spectrophotometer has been assembled, and allows precise measurement of oxygen equilibria of purified hemoglobin solutions. The effects of each of the cellular factors which are known to modify hemoglobin function can be investigated systematically (i.e., temperature pH, salt, 2,3-DPG, and hemoglobin concentration) with hemoglobin which has been purified by column chromatographic methods.

2) A new apparatus for the precise measurement of whole blood oxygen equilibria which has been developed by Drs. Berger (NHLI, Laboratory of Technical Development) and L. Rossi-Bernardi (University of Milan) is currently operating in our laboratory.

3) A rapid, convenient method for the identification of mutant human hemoglobins has been developed and employs cellulose thin layers for "fingerprint" analysis, using extremely small samples and minimal space and time.

Major Findings: 1) A hemoglobin variant (hemoglobin McKees Rocks β145→Term) has been characterized in regard to structure and function. It is the first mammalian protein whose structural alteration has arisen by mutation which corresponds to a "nonsense" mutation in bacteria: two amino acids are lacking



700

at its carboxyl terminal end. It has the highest oxygen affinity of any known human variant, yet carriers of it are asymptomatic.

2) The properties of the blood of sheep and goats after an anemia-induced "switch" to hemoglobin C is currently under study. We have demonstrated that after the switch, goat blood has an extraordinary sensitivity to $CO_2$, such that it promotes the unloading of oxygen. A correlation between this unique property and the known structure of goat hemoglobin is underway.

Significance to Biomedical Research and Institute Programs: The control of the respiratory function of blood is important in any condition in which tissue oxygen delivery may be compromised. In addition, the capacity of the blood to deliver oxygen plays an important role in the compensatory physiology of anemia and the regulation of the size of the red cell mass.

Proposed Course of Project: 1) A systematic characterization of the properties of normal blood will be continued; the availability of the new equipment renders existing data obsolete.

2) Additional human hemoglobin variants may be studied. Of special interest is the compensatory mechanisms which are employed by subjects with high affinity hemoglobins.

3) The characterization of the role of hemoglobin C in the adaptation of sheep and goats to their environment will be pursued. Other animals with unique environmental demands might be studied also.

4) New equipment is being designed by the Laboratory of Technical Development (Dr. Berger) which will enable the study of the kinetic properties of blood oxygenation and deoxygenation. It is hoped that this study will lead to better understanding of the relationships between capillary blood flow and oxygenation which occurs in vivo.

Honors and Awards: None

Publications: None

Project No. Z01 HL 02211-01 MHB
1. Molecular Hematology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Evaluation of Alteration in Blood Oxygen Affinity as a Basis for the Treatment of Sickle Cell Anemia

Previous Serial Number: None

Principal Investigator: Robert M. Winslow, M.D.

Other Investigators: Jack Fulmer, M.D.
Arthur W. Nienhuis, M.D.
Ronald G. Crystal, M.D.
Mei-Lei Swenberg, Ph.D.

Cooperating Units: Pulmonary Branch, NHLI
Laboratory of Technical Development, NHLI

Project Description:

Objectives: This project is designed to study the relationship between sickling and the oxygen transport of blood. Sickling is a function of blood oxygen saturation and is therefore affected by factors which modify oxygenation. The overall objective is to define the extent of organ involvement and some parameters of blood oxygen transport (pulmonary functions, response to exercise, blood oxygen affinity, and sickling) in a small number of patients with sickle cell anemia. The effects of agents which modify one or more of the known properties of blood oxygenation or sickling can then be investigated. The current protocol is designed to test the effect of low dose carbon monoxide on sickling and exercise tolerance. In addition, in vitro studies are directed toward an understanding of the molecular and cellular mechanisms which regulate oxygenation and gellation of hemoglobin within sickled cells.

Methods: The life span of red cells is measured by labelling a cohort of reticulocytes with radioactive amino acids. The differential response of young and old cells to a therapeutic maneuver can then be observed by labelling a second cohort with a different label ($^{14}C$) or ($^{3}H$) after an appropriate interval. A battery of clinical tests is administered which defines the degree of organ involvement in individual patients. Blood oxygen affinity is measured using new equipment designed in the Laboratory of Technical Development, NHLI. Pulmonary functions and exercise testing are carried out in collaboration with the Pulmonary Branch, NHLI.

Major Findings: 1) A relationship has been found between the number of irreversibly sickled cells (ISC's) degree of anemia, and blood $P_{50}$: patients with the most ISC's frequently have low hematocrits and high $P_{50}$'s. These findings suggest that ISC's have low oxygen affinity and in spite of their obvious rheologic disadvantage may facilitate oxygen delivery.

2) There is very poor correlation between the subjective assessment of the

severity of symptoms of sickle cell anemia and the objective measurements of organ damage.

Significance to Biomedical Research and Institute Program: Sickle cell anemia is a serious genetic disease which has a long history of therapeutic failure. Before rational therapies can be undertaken, greater understanding of pathophysiology is required. Little attention has been paid to the relationships between sickling and oxygen supply and demand and the effect of a given therapy thereon.

Proposed Course of Project: 1) We plan to continue the Clinical Protocol and study the effect of the alteration of blood oxygen affinity on sickling, by the administration of carbon monoxide in low doses to selected patients.

2) We plan to investigate the mechanism of the low oxygen affinity of ISC's and to attempt to understand whether these unique cells are harmful or beneficial in sickle cell anemia patients.

3) A model of the respiratory function of normal blood will be built up and applied to the problem of sickle cell anemia. To this end, the kinetic and equilibrium oxygenation properties of sickle blood will be carefully studied, using the new equipment described above.

Honors and Awards: None

Publications: None

Project No. Z01 HL 02203-03 MHB
1. Molecular Hematology Branch
2. Clinical Hematology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Regulation of Hemoglobin Chain Synthesis in Beta-Thalassemia

Previous Serial Number: NHLI-109(c)

Principal Investigator: Arthur W. Nienhuis, M.D.

Other Investigators: Ramon Velez, M.D.
Judith Kantor, Ph.D.
Alan Steggles, Ph.D.
Golder Wilson, M.D., Ph.D.
W. French Anderson, M.D.

Cooperating Units: None

Project Description:

Objectives: Beta-thalassemia, also known as Cooley's Anemia, is an hereditary disease characterized by severe anemia. The anemia is a consequence of a low level of beta chains of hemoglobin being produced by the patient's red blood cells. This results in an excess of alpha globin chains. These excess alpha chains precipitate in the cell and lead to the destruction of the cell. No amino acid change has been found in the hemoglobin beta chains of thalassemic patients. It is generally assumed, therefore, that the molecular abnormality resides in the regulation of beta chain synthesis. Analysis of globin mRNA from thalassemia cells has indicated that there is a reduced amount of beta globin mRNA relative to that for the alpha globin, so that the defect must be specifically in the production of messenger RNA. The normal mechanisms for regulation of globin mRNA production and the nature of the defect in beta thalassemia cells are being investigated.

Methods: Reticulocytes and/or bone marrow are obtained both from patients with beta-thalassemia and from patients displaying a high reticulocyte count secondary to another cause (for example, recovery from folic acid deficiency, sickle cell anemia, etc). Globin mRNA is isolated from reticulocytes and used as a template for synthesis of globin complementary DNA (cDNA) by the viral enzyme, RNA directed DNA polymerase. Alpha and beta globin cDNAs are made using mRNA from reticulocytes of patients with beta thalassemia and hemoglobin H disease, respectively, and these are further purified by hydroxylapatite chromatography. DNA and nuclear RNA are isolated from cells of normal patients and those with beta thalassemia and the relative amounts of alpha and beta nucleotide sequences in these components is determined. Chromatin is utilized for cell-free synthesis of globin mRNA.

Major Findings: 1) DNA from beta thalassemic cells hybridized equally to both alpha and beta human cDNA in a manner quantitatively similar to the hybridization of these probes to normal human DNA. Hence, gene deletion does not appear to be responsible for the deficiency of beta globin mRNA production in the patients we have studied.

2) Methodology has been established for the isolation of high molecular weight nuclear RNA from a small number of bone marrow cells. Application of these techniques to thalassemia cells will permit determination of the relative number of alpha and beta sequences in the immediate product of transcription.

Significance to Biomedical Research and Institute Program: The thalassemias are severe hereditary diseases which as a group affect more individuals world wide than any other single gene defect. A method is needed for treating these diseases which will reduce or eliminate the frequent blood transfusions that are often required. In addition, the techniques utilized to learn how the rate of hemoglobin synthesis is regulated can then be applied to studying other diseases where the defect is also in faulty regulation in the synthesis of a gene product.

Proposed Course of Project: The relative amount of alpha and beta globin messenger RNA sequences synthesized from thalassemic and non-thalassemic chromatin in the cell-free RNA synthesizing system will be quantitated. Fractionation of the nuclear components of thalassemic erythroid cells will be carried out to determine the molecular defect in thalassemia.

Keyword Descriptors: Thalassemia, globin messenger RNA, globin genes, nuclear RNA, nucleic acid hybridizations, anemia, gene regulation, bone marrow cells, reticulocytes.

Honors and Awards: None

Publications:

1. Anderson, W.F.: Isolation and translation of messenger RNA from beta-thalassemia red cells. Ann. N.Y. Acad. Sci. 232:15-32, 1974.

2. Nienhuis, A.W., and Anderson, W.F.: The molecular defect in thalassemia. Clinics in Haematology 3:437-466, 1974.

3. Velez, R., Kantor, J.A., Picciano, D.J., Anderson, W.F., and Nienhuis, A.W.: Alpha and beta complementary DNAs of human and rabbit: Specificity of hybridization. J. Biol. Chem. in press.

Project No. Z01 HL 02204-03 MHB
1. Molecular Hematology Branch
2. Clinical Hematology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Mechanism of Hemoglobin Switching in Sheep and Goats

Previous Serial Number: NHLI-287

Principal Investigators: Jane Barker, Ph.D.
Arthur W. Nienhuis, M.D.

Other Investigators: Thomas Musliner, M.D.
Joseph E. Pierce, M.D.
Leonard Stuart
W. French Anderson, M.D.

Cooperating Units: Section on Laboratory ANimal Medicine and Surgery, NHLI
Ungulate Unit, NIH Animal Center

Project Description:

Objectives: This project utilizes the erythropoietin-induced switch in hemoglobin phenotype which occurs in sheep and goats as a model system for understanding factors which regulate globin gene expression in erythroid cells. Both the cellular events accompanying differentiation and hemoglobin synthesis and the subcellular events related to the regulation of transcription will be explored.

Methods: Bone marrow is obtained from normal, anemic, or transfused phlethoric animals. The cells are grown in vitro, directly or after fractionation by unit gravity sedimentation, under conditions in which erythroid colonies form. Globin synthesis is quantitated and the type and amount of each globin chain determined by chromatography or gel electrophoresis. Chromatin extracted from bone marrow is assayed in vitro for its ability to support synthesis of globin gene sequences as measured by nucleic acid hybridization to complementary DNA

Major Findings: 1) Cells capable of generating erythroid colonies are large, presumably immature cells and may be separated from the differentiating erythroid cells by unit gravity sedimentation. These colony forming cells respond to high concentrations of erythropoietin by producing colonies active in the synthesis of hemoglobin C. Initial results indicate that a fraction of colony forming cells of intermediate sedimentation velocity have the greatest potential for hemoglobin C production. This differential sensitivity to erythropoietin may reflect the position of the cell in the erythroid maturation sequence.

2) Sheep colony forming cells appear to require an approximately ten-fold higher concentration of erythropoietin for stimulation of hemoglobin C production than do goat cells. However, colony formation occurs at equivalently low erythropoietin concentrations for the cells of both species.

3) Human erythroid colonies have been grown from bone marrow of patients with various hemolytic anemias. Thus, the concepts and techniques developed for study of the A to C switch in the cells of sheep and goats, may prove useful in studying regulation of human hemoglobin synthesis.

Proposed Course of the Project:

The marrow fractionation studies will be extended to determine if a population of precursor cells can be obtained which is capable of generating colonies producing only hemoglobin C. The relationship between the duration of exposure to high concentration erythropoietin and the production of hemoglobin C will be defined to determine if commitment to hemoglobin phenotype is an irreversible event. Synthesis of specific hemoglobins in human colonies will be examined.

Significance to Biomedical Research and Institute Program: Activation of hemoglobin F synthesis in the cells of patients suffering from sickle cell anemia or thalassemia should totally alleviate the symptoms of the disease. The sheep and goat model system will be useful in defining conditions under which hemoglobin phenotype can be influenced thereby facilitating the search for agents which regulate the proportion of fetal hemoglobin synthesis in human erythroid marrow cells.

Keyword Descriptors: Erythropoieses, regulation of hemoglobin synthesis, erythropoietin, erythroid colony, bone marrow fractionation, human bone marrow *in vitro*, globin gene regulation.

Honors and Awards: None

Publications:

1. Nienhuis, A.W., Elson, N.A., Barker, J.E., and Anderson, W.F.: Hemoglobin switching in sheep and goats: Aspects of the molecular mechanism. Ann. N.Y. Acad. Sci. 241: 566-581, 1974.

2. Nienhuis, A.W., and Bunn, H.F.: Hemoglobin switching in sheep and goats: Occurrence of the hybrid hemoglobin, $\alpha_2\beta^A\beta^C$ in the same red cell. Science 185:946-948, 1974.

3. Barker, J.E., Anderson, W.F., and Nienhuis, A.W.: Hemoglobin switching in sheep and goats: V. Effect of erythropoietin concentration on *in vitro* erythroid colony growth and globin synthesis. J. Cell Biol. 64:515-527, 1975.

4. Nienhuis, A.W., Barker, J.E., Deisseroth, A., and Anderson, W.F.: Regulation of globin gene expression. In Weatherall, D.J. (Ed): Disordered Erythropoieses in Childhood. CIBA Symposium, in press.

Project No. Z01 HL 02208-02 MHB
1. Molecular Hematology Branch
2. Clinical Hematology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Iron Chelation Therapy in Transfusional Hemosiderosis

Previous Serial Number: NHLI-95(c)

Principal Investigator: Arthur W. Nienhuis, M.D.

Other Investigators: Walter Henry
Frederic Bartter, M.D.
Roger Aamodt, M.D.

Cooperating Units: Cardiology Branch, NHLI
Endocrinology Branch, NHLI
Section on Total Body Counting, Nuclear Medicine Department

Project Description:

Objectives: The object of this study is to evaluate available iron chelators, to maximize their effectiveness, and to test new chelators as they become available. The current protocol is designed to evaluate the effect of Desferal on total iron balance. The added effect of ascorbic acid on urinary iron excretion and GI absorption is also being evaluated.

Methods: Patients are studied under metabolic balance conditions. A standard diet of measured calcium, phosphorus, and iron content is eaten daily and the total excretion of these elements is measured in the stool and urine. Analysis of iron absorption is obtained by administration of tracer doses of Fe-59 and measurement of absorption by the total body counter.

Major Findings: 1) Ascorbic acid causes a marked increase in urinary iron excretion in younger, less heavily iron-loaded patients. Iron absorption and the excretion of iron in the stool are unchanged. Thus negative iron balance in response to desferal is markedly enhanced by ascorbic acid in these patients.

2) Older, more heavily iron-loaded patients, treated with desferal, show a more varied response to ascorbic acid. One patient showed a doubling of urinary iron excretion, but an equivalent reduction in stool iron, so that net iron balance was unaffected by ascorbic acid. Withdrawal of ascorbic acid was followed by a fall in the urinary iron over several weeks which paralleled a reduction in white cell ascorbic acid to subnormal levels. Thus, the effect of ascorbic acid administration in this patient is apparently related to its intracellular concentration and raises the possibility that the tissue source of chelated iron may also be influenced by ascorbic acid. Another patient showed an increase in both urinary and fecal iron excretion in response to ascorbic acid. Hence net negative iron balance was markedly increased.

3) A clinical pathological correlative study was performed on a 23 year old patient with congenital anemia, transfused from birth, who died from cardiac hemachromatosis. The biochemical form of iron was found to be different for various tissues; ferritin iron was concentrated in the liver and the pancreas, while the metabolically less active form, hemosiderin was concentrated in the heart and endocrine tissues. Heavy deposits of iron were present in all the endocrine glands although function was variably affected. Parathyroid function was abolished but the plasma concentrations of several of the pituitary hormones was elevated.

4) Echocardiographic studies were performed on several iron-loaded patients at the NIH, and also at two large thalassemia clinics in New York City. Left ventricular wall thickening and an increase in cardiac weight presumably reflected myocardial iron deposition and was present in even the youngest patients, despite lack of clinical evidence of cardiac hemachromatosis. These data will be useful in the subsequent evaluation of chelation therapy.

Significance to Biomedical Research and Institute Program: Transfusional 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: This 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.

Keyword Descriptors: Hemachromatosis, cardiac hemosiderosis, iron chelation, Desferal, ascorbic acid, ferritin, hemosiderin, metabolic balance study, endocrine gland dysfunction, thalassemia, echocardiogram.

Honors and Awards: None

Publications:

1. Arnett, E.N., Nienhuis, A.W., Henry, W.L., Ferrans, V.J., Redwood, D.R., and Roberts, W.C.: Massive myocardial hemosiderosis: A structure-function Conference at the National Heart and Lung Institute. Am. Heart J. in press.

Project No. Z01 HL 02209-02 MHB
1. Molecular Hematology Branch
2. Clinical Hematology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Cardiac Hemolytic Anemia.

Previous Serial Number: NHLI-96(c)

Principal Investigator: Arthur W. Nienhuis, M.D.

Other Investigators: Gary Weiss, M.D.
Laurence Corash, M.D.
Harvey Gralnick, M.D.
Roger Aamodt, M.D.

Cooperating Units: Cardiac Surgical Branch, NHLI
Clinical Pathology Branch, NIH Clinical Center
Section on Total Body Counting, Nuclear Medicine Department.

Project Description:

Objectives: The objectives of this study are:

1) to evaluate various parameters of hemolysis as to their usefulness in measuring hemolytic rate in patients with cardiac hemolytic anemia;

2) to determine the state of iron balance in these patients when on therapeutic doses of oral iron; and

3) to seek ways to minimize the rate of hemolysis in patients with cardiac lesions, or prosthetic valves.

Methods: Patients are admitted to the Clinical Center for the following studies: red cell chromium survival, carbon monoxide red cell survival, iron absorption and urinary iron excretion. Cardiac output is estimated with the echocardiogram.

Major Findings: 1) The degree of anemia has no consistent effect on the hemolytic rate in patients with cardiac hemolysis. Thus, certain patients show reduced rate of red cell destruction following transfusion while others show no change in hemolytic rate.

2) Several patients have been shown to have mild or moderate hemolysis but severe anemia, reflecting inadequate bone marrow compensation. Elevated sedimentation rate, elevated gamma globulins and poor response to oral iron suggests that a chronic inflammatory process may be responsible for relative bone marrow failure, although intensive diagnostic evaluation has defined such a process in only two or seven patients.

Significance to Biomedical Research and Institute Program: Cardiac hemolytic anemia is a significant complication of prosthetic valve replacement. To maximize the clinical benefit of this surgical procedure to these patients efforts must be made to minimize the severity of the anemia.

Proposed Course of the Project: Patients currently followed by the Hematology Branch will be re-evaluated periodically, in an effort to identify factors responsible for the inadequate bone marrow response to hemolysis. Additional patients will be studied to define further the relationship between hemolytic rate, degree of anemia, and bone marrow function.

Keyword Descriptors: Cardiac hemolytic anemia, valve prosthesis, red cell survival, bone marrow function, iron absorption, valvular heart disease.

Honors and Awards: None

Publications: None.

Annual Report of the
Section on Pulmonary Biochemistry
Pulmonary Branch
National Heart and Lung Institute
July 1, 1974 through June 30, 1975

The connective tissue of the lung is fundamental to lung structure and function. In human pulmonary disease there is a broad spectrum of connective tissue involvement from an apparent excess of connective tissue in the interstitial lung disorders to an apparent destruction of the connective tissue frame work in the emphysematous disorders. The overall objective of this work is to understand and eventually control the mechanisms of lung connective tissue synthesis and destruction so that ultimately we can selectively inhibit or reverse the fibrotic process in the interstitial disorders and promote the preservation and generation of functional alveolar units in the emphysematous disorders. The initial objectives of this laboratory since its inception in July 1972 have been to: (1) develop the in vitro technology by which these problems could be attacked; (2) set up a clinical chest service to evaluate patients with connective tissue related lung disorders; (3) apply the in vitro technology to the study of these disorders in animal models and in human disease. All three objectives have been achieved. Specifically, it is now possible to examine the synthesis and proteolysis of connective tissue in explant cultures of animal and human lung, in diploid cell lines derived from animal and human lung and in homologous and heterologous cell-free protein synthesizing systems. A clinical pulmonary service has been established using sophisticated methods to evaluate patients with connective tissue related lung disorders. In many instances, the methods of explant culture, cell culture and cell-free protein synthesis can be used to evaluate these clinical problems. The following is a summary of the ongoing projects in the laboratory this past year.

I. Models of Lung Growth

Two models are being used to investigate the control of synthesis and proteolysis of lung connective tissue components: (1) changes during normal lung growth and (2) "compensatory" lung growth following unilateral pneumonectomy.

Just prior to birth, approximately 3-5% of the total amino acids incorporated into lung protein per hour go into collagen. In the neonatal period, there is a shift in the emphasis of the proteins synthesized by lung so that this figure goes to 12-15%. During this period there is a rapid accumulation of the total amount of collagen in the lung as well as the relative amount of collagen per unit lung mass. By adulthood, the level of collagen synthesis returns to its low level of 3-5% and the accumulation of collagen in the lung stops. Within one month after left pneumonectomy in the adult rabbit, the mass of the right lung doubles. This growth can be characterized by a period of rapid collagen accumulation preceded by a shift in emphasis of total protein synthesis toward collagen synthesis. As a result, the total amount of collagen doubles, but the density of collagen remains constant. This is in

contrast to normal neonatal lung growth in which the shift in protein synthesis toward collagen accumulation results in an increased concentration of collagen. Studies with mechanical alteration of the chest cavity following unilateral pneumonectomy have suggested that mechanical factors may be partially responsible for the initiation of the complex biochemical events associated with lung growth.

The adult rabbit lung synthesizes approximately 1 mg of collagen per day, yet the amount of collagen per unit lung mass does not change. Thus the synthesis of collagen must be matched by an equivalent proteolysis of collagen so as to prevent abnormal collagen accumulation. Methods have been developed to define the degradation of newly synthesized lung collagen as early proteolysis (0-48 hours) and late proteolysis (greater than 48 hours). In the newborn, 25% of newly synthesized collagen is degraded within four hours, but there is no further proteolysis of this collagen over the next two days. In the adult, although synthesis of collagen is 2-3 fold less, the same percentage of collagen is degraded in the same time. Pharmacologic agents, such as colchicine can significantly increase the amount of early proteolysis of newly synthesized lung collagen. Thus, it appears that (1) 25% of newly synthesized lung collagen is degraded very rapidly; (2) early proteolysis of newly synthesized lung collagen does not appear to be important in controlling collagen accumulation in lung growth; (3) most likely, late proteolysis is responsible for the degradation of the remainder of the newly synthesized lung collagen; and (4) the proteolysis of collagen can be significantly altered by pharmacologic agents. The latter may become very important in therapeutic intervention of the fibrotic lung disorders.

Although the glycosaminoglycans contribute less than 1% to total lung dry weight, they are potentially of major importance in lung growth and disease because of their influence on connective tissue accumulation and physical state. There are significant changes in glycosaminoglycans as the lung grows. For example, chondroitin 4-sulfate is relatively more important in the fetal lung while dermatan sulfate, heparin and heparan sulfate become significant in adult life. These alterations in lung glycosaminoglycan composition are, in part, controlled by age related changes in glycosaminoglycan synthesis. These changes may have importance in controlling alterations in lung function during development.

II. Control of Collagen Synthesis in Lung Cell-Free Systems

An active, partially fractionated cell-free protein synthesizing system has been established from rabbit lung. Lung polysomes can also be translated in heterologous cell-free systems including the rabbit reticulocyte and wheat embryo systems. Collagen in lung is synthesized on the class of large polysomes associated with the endoplasmic reticulum. During lung development there are significant changes in the relative proportion of lung polysomes that synthesize collagen suggesting that at least some of the control of lung collagen synthesis may be due to changes in lung collagen gene expression or to changes in the number of cells that synthesize collagen. These methods are being applied to tissue culture systems of animal and human lung cells utilized in <u>in vitro</u> models of fibrotic lung disease.

The cell-free system is currently being utilized to identify vulnerable steps in the process of connective tissue synthesis which may be influenced to treat specific lung disorders.

III. Heterogeneity of Lung Collagen

Collagen is the most abundant protein in lung, comprising 10-15% of the adult lung. This collagen is heterogeneous; there are probably four different types of collagen synthesized by the lung. Using animal and human lung and cultured lung cells, the following has been determined:

The lung parenchyma and blood vessels synthesize $\alpha 1(I)$ and $\alpha 2$ chains (Type I collagen) and $\alpha 1(III)$ chains (Type III collagen), while the tracheobronchial tree synthesizes $\alpha 1(II)$ chains (Type II collagen). These collagen chains have distinct primary amino acid sequences as demonstrated by cyanogen bromide peptide mapping techniques. All evidence to date suggests that while lung collagen is markedly heterogeneous, each collagen type is identical to collagen elsewhere in the body (e.g., Type I is the same from lung, skin, bone, tendon, blood vessels; Type II is the same from tracheobronchial tree and sternal cartilage; and Type III is the same from lung, aorta and skin).

The fibrotic lung diseases have many primary stimuli including inhalation of toxic materials, hypersensitivity states, radiation injury and associated with systemic disorders such as scleroderma. It is likely that these disorders are associated with injury to different lung cells, suggesting the possibility that there may be a heterogeneity in the types of collagen synthesized in these different diseases. It may be possible to classify the interstitial lung diseases on the basis of the types of collagen synthesized in a similar fashion to the lipoprotein disorders and certain hemoglobin disorders.

IV. Collagen Synthesis in Cultured Lung Cells

The application of biochemical technology to human lung disease is limited by the complexity of the organ and the unavailability of large quantities of lung cells from patients with lung disease. There are more than 40 cell types in lung, and some of these cells are responsible for the synthesis of 5 different types of collagen chains. When the lung synthesizes abnormal amounts of collagen in response to injury, the same cells normally producing collagen may proliferate; they may synthesize relatively more collagen or cells that do not normally synthesize collagen may be "turned on" to do so. One of the primary objectives of this laboratory is to develop the technology to culture the cells from lung that are important in collagen synthesis in health and disease.

Fibroblasts from animal and human lung synthesize both Type I and Type III collagen. An epithelial cell cultured from fetal cat lung also synthesized Type I and Type III collagen but not as actively as the fibroblast. Pulmonary alveolar macrophages do not synthesize collagen. Methods have been developed to use molecular weight maps of the proteins secreted by these cells

as specific "fingerprints" of each cell type. The "fingerprint" of the fibroblast,epithelial cell and macrophage are markedly different.

These methods are being adapted to establish model systems of human lung diesase in which the interaction of human or animal lung cells, alveolar macrophages, lymphocytes and serum components can be studied in vitro.

V. The Accumulation of Collagen in the Human Lung

The in vitro technology has been established to quantitate human lung collagen composition and synthesis from biopsy specimens in normal and diseased lungs. Normal human lung parenchyma synthesizes at least two collagen chains ($\alpha$1(I), $\alpha$2) and probably a third ($\alpha$1(III)). Fibroblasts cloned from human fetal lung synthesize $\alpha$1(I), $\alpha$2 and $\alpha$1(III) chains. In the 16-20 week human fetal lung and in the adult human lung, approximately 4% of the total amino acids incorporated into protein are incorporated into collagen. Biopsies from three patients with idiopathic pulmonary fibrosis have been examined and, interestingly, none had increases in the percentage of protein synthesis that was collagen, suggesting that the molecular pathology in fibrotic lung disease is more than simply an increase in fibrous tissue synthesis.

Methods developed in animal systems to quantitate collagen proteolysis are being utilized with human lung biopsies to establish the significance of control of collagen degradation on human lung collagen accumulation. These techniques are adaptable to quantitating the influence of pharmacologic agents on the fibrotic process and thus predicting, for each patient, which agents are most efficacious.

VI. Experimental Models of the Interstitial Lung Disorders

A major goal of this laboratory is to understand and eventually control the mechanisms involved in connective tissue synthesis and degradation in human lung disease involving these components. Because the availability of human lung biopsy material is limited, it necessitates that any biochemical techniques applied to this tissue is worked out first in animal models, thus insuring the maximum amount of information and the maximum benefit to the patient. For that reason two groups of models of fibrotic diseases are being developed: (1) animal models; and (2) tissue culture models.

Animal models - Although collagen accumulation takes place in silica exposed animals, it is a difficult model to work with because the silicotic "nodules" are discrete and inhomogeneous. Radiation induced fibrosis has been a much more satisfactory model and preliminary studies have demonstrated increased collagen density 6 weeks after 6000 R to the lung.

Tissue culture models - The pulmonary alveolar macrophage can be induced to secrete collagenase, a unique and specific enzyme for mammallian collagen. Rabbit macrophages activated with BGC (in vivo) will secrete collagenase after 1-2 days in culture. Exposure of these macrophages to protein antigens derived from mycobacterium will further augment this finding. Sufficient

quantities of mammallian collagenase are being purified to produce antibodies specific for it. Soon, it should be possible to have tissue culture systems involving lung cells, macrophages, lymphocytes and serum from animals, normal humans and humans with fibrotic lung disease in which the rates of synthesis and degradation of specific collagen types can be quantitated and manipulated.

VII. Studies of Patients with Fibrotic Lung Disease

The fibrotic lung disorders represent 15-20% of the non-infectious disorders of lung. The mean survival of these patients is 45 months from the onset of symptoms. Although approximately 5-10% of these patients respond to corticosteroids, there is essentially no treatment for the remainder. The natural history, etiology and pathogenesis of "idiopathic" fibrotic lung disease is poorly studied. The Pulmonary Branch, NHLI, has undertaken a detailed study of these patients with several major objectives:

(1) To determine the etiology of idiopathic fibrotic lung disease.
(2) To follow patients longitudinally to determine (a) the natural history of this disorder and (b) which pulmonary function parameters are most sensitive to the disease process.
(3) To correlate pulmonary alveolar constituents (fluid and pulmonary alveolar macrophages) with fibrotic disease.
(4) To correlate radioisotopic monitors of lung function (ventilation, perfusion and gallium scans) with the disease process.
(5) To correlate lung pathogenic alterations with biochemical and functional changes.
(6) To study the pharmacologic therapy of this disease process.

Preliminary studies suggest:

(1) Obstruction to airflow is a significant part of the pathology of this disorder.
(2) Classic immune mechanisms (at least as defined by serologic parameters) are not frequent occurrences.
(3) Rare patients can be classified as having environmental etiologies (defined by electron probe analyses of biopsies).
(4) The biochemical pathology is much more than simply an increase in connective tissue synthesis.
(5) Ventilatory and arterial blood gas parameters with exercise are probably the most sensitive monitors of the disease process.
(6) Patients with this disorder have marked abnormalities in ventilation-perfusion mismatching.

These disorders are almost uniformly fatal and affect a significant proportion of the population. Up to this time, there has been no information on pathogenesis and there is essentially no cure. By combining studies on patients with these disorders with our large basic research program in the control of connective tissue accumulation in lung, we expect to make major inroads into understanding and eventually curing these disorders.

Project No. Z01 HL 02401-03 PB
1. Pulmonary Branch
2. Section on Pulmonary Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Models of Lung Growth

Previous Serial Number: NHLI 110

Principal Investigators: Ronald G. Crystal, M.D.
Morton J. Cowan, M.D.
Allen L. Horwitz, M.D.
Robert Bienkowski, Ph.D.

Other Investigators: None

Cooperating Units: William M. Thurlbeck, M.D.
Midhurst Medical Research Institute
Midhurst Sussex, England

Project Description:

Objectives: The major function of the lung is to transfer oxygen from the atmosphere to the blood and carbon dioxide from the blood to the atmosphere. This function is provided by the unique structure of lung and its mechanical properties in relation to changes in intrapleural pressure. The structure and mechanical properties of lung are intimately dependent on the connective tissue composition of the lung. The presence of these connective tissue components is controlled by a balance of synthesis and degradation. Two models are being used to investigate the control of connective tissue synthesis and degradation: (1) changes during normal lung growth and (2) "compensatory" lung growth following unilateral pneumonectomy.

Methods: (1) Connective tissue synthesis and degradation during lung development. Methods have been developed to keep explants of lung tissue viable for more than 24 hours under defined culture conditions. Minces from rabbit lungs obtained at different ages are incubated at varying times and the rates of collagen synthesis, non-collagen protein synthesis and glycosaminoglycan synthesis are determined. In parallel studies, the density of lung collagen, total glycosaminoglycans and specific types of glycosaminoglycans are quantitated. Other methods are used to quantitate the fraction of newly synthesized collagen that is subsequently degraded.

(2) Lung growth following pneumonectomy. Adult rabbits undergo unilateral pneumonectomy while paired litter mate controls undergo (a) thoracotomy

without pneumonectomy or (b) pneumonectomy and immediate filling of the empty chest cavity with wax. At intervals following surgery, the lungs are examined in explant culture as described above.

Major Findings: (1) Connective tissue synthesis and degradation during lung development. Last year the normal pattern of lung collagen and non-collagen protein synthesis were described in normal rabbit lung growth (see individual project report # NHLI 110). The collagen synthesis methods have been extended so the lung explants can be kept viable for more than 24 hours. This has permitted examination of the early proteolysis (0-24 hours) of newly synthesized lung collagen. In the newborn lung, 25% of the collagen synthesized in four hours is degraded to less than 10,000 molecular weight ( from original 100,000 molecular weight). Under the conditions used, there is no further degradation of newly synthesized collagen from 4 to 24 hours of incubation. In the adult lung, although synthesis of collagen is 2-3 fold less, 25% of the newly synthesized collagen is degraded. Thus it appears that: (a) one-fourth of newly synthesized lung collagen is degraded very rapidly (probably intracellularly or immediately following secretion from the cell); (b) this mechanism does not appear to be important in the control of lung collagen accumulation during lung growth; and (c) since collagen density does not change in the adult, 75% of the collagen synthesized by the adult lung must be present for greater than 24 hours before it is degraded.

The density of lung glycosaminoglycans (GAG) in lung varies significantly with age. Parenchymal GAG content ranged between 0.2 - 0.4% (w/w) of dry weight with highest density in adult lung. Fetal lung contains a relatively high proportion of chondroitin 4-sulfate while the GAG in lung parenchyma of older animals was predominantly dermatan sulfate, heparan sulfate and heparin. The rate of synthesis of total GAG per cell increased with development to a maximum in lung from weanling rabbits and fell to low rates of synthesis in mature rabbits. Fetal rabbit lung parenchyma synthesized mostly hyaluronic acid and heparan sulfate while in weanling rabbit parenchyma hyaluronic acid and chondroitin 4/6 sulfate synthesis was greatest. In mature animals, the rates of synthesis of all types of GAG were relatively low, but there is a relatively greater emphasis on synthesis of dermatan sulfate and heparin.

(2) Lung growth following pneumonectomy. Within one month after left pneumonectomy in the adult rabbit, the mass of the right lung doubles. This growth can be characterized by a period of rapid collagen accumulation preceded by a shift in emphasis of total protein synthesis toward collagen synthesis. As a result, the total amount of collagen doubles but the concentration of collagen remained constant. This is in contrast to neonatal lung growth in which the shift in protein synthesis toward collagen accumulation resulted in an increased concentration of collagen.

When wax is used to obliterate the space left by pneumonectomy, the "compensatory" lung growth ( changes in lung cells, collagen synthesis and accumulation) is completely obviated. Hence, it appears that mechanical factors (i.e., size of the chest cavity) are crucially important in determining lung

growth in this model.

Significance to Biomedical Research and Institute Program: These two models provide a means to study the lung during periods of "turning off" or "turning on" of connective tissue synthesis and degradation. Through a comprehensive approach at the cellular and sub-cellular levels, it should be possible to understand the control of structural and non-structural protein synthesis in the lung. This has obvious applications to the control of fibrosis in the interstitial lung diseases. An understanding of lung growth will hopefully provide means to approach the regeneration of functional alveolar units in the emphysematous disorders.

Proposed Course to Project: The methods to quantitate the density, synthesis and early degradation of lung collagen and the density and synthesis of lung glycosaminoglycans in models of lung growth are now complete. The next steps include:

(1) Quantitation of "late" degradation of collagen present for more than 24 hours. Investigation into manipulation of lung collagen degradation (both "early" and "late") with serum and pharmacologic agents.

(2) Continued investigation into the factors responsible for the growth of the lung following unilateral pneumonectomy. There is preliminary evidence that serum "growth" factors, as yet undefined, may play an important role.

(3) Continued investigation of the subcellular mechanisms involved in the control of the synthesis of connective tissue components during lung growth (see report on mechanisms of collagen and non-collagen protein synthesis in lung cell-free systems, project report no. Z01 HL 02402-03 PB).

(4) Investigations into the interactions of connective tissue components (collagen, elastin, glycosaminoglycans) between themselves and lung cells in controlling lung connective tissue accumulation.

(5) Role of immunologic and proteolytic mechanisms (lymphocytes, macrophages, immunoglobulins, complement) in the maintenance of normal lung connective tissue.

(6) Quantitate the morphometric changes in post-pneumonectomy lung growth (with Dr. William Thurlbeck, Midhurst Medical Research Institute, Midhurst Sussex, England).

Keyword Descriptors: Lung, Collagen, Glycosaminoglycans, Pneumonectomy, Lung Growth, Proteolysis, Explant Culture.

Honors and Awards: None

Publications:

Crystal, R.G. Lung Collagen: Definition, Diversity and Development. Fed. Proc. 33: 2248, 1974.

Cowan, M.J. and Crystal, R.G. Lung Growth After Unilateral Pneumonectomy: Quantitation of Collagen Synthesis and Content. Am. Rev. of Resp. Dis. 111: 267, 1975.

Cowan, M.J., Collins, J.F. and Crystal, R.G. Collagen and Lung Growth: A Prototype of Connective Tissue Differentiation. In: J. Last (Ed.): Eukaryotes at the Subcellular Level: Development and Differentiation. M. Dekker, New York, 1975 (in press).

Project No. Z01 HL 02402-03 PB
1. Pulmonary Branch
2. Section on Pulmonary Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 3, 1974 through June 30, 1975

Project Title: Control of Collagen Synthesis in Lung Cell-Free Systems

Previous Serial Number: NHLI 113

Principal Investigators: Ronald G. Crystal, M.D.
James F. Collins, Ph.D.
Morton J. Cowan, M.D.

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: While explant culture systems are adequate for the description of protein synthesis and degradation by lung, it does not have the potential to understand the molecular control of these events. Since a primary goal of this laboratory is to understand and eventually control the mechanisms of lung connective tissue accumulation, it is necessary to develop systems in which specific identification of control mechanisms can be identified. For this reason, a cell-free protein synthesizing system is desirable and affords an opportunity to understand control of lung protein synthesis at several levels.

Methods: The components necessary for a partially fractionated cell-free protein synthesizing system have been isolated from rabbit lung and human lung fibroblast cultures using conventional techniques. These components include polyribosomes, 0.5M KCl polysome wash fraction (including initiation factors, elongation factors, synthetases, and tRNA) and post-translational enzymes (e.g., prolyl hydroxylase). Polysomes are separated into two classes: those bound to the endoplasmic reticulum and those free in the cytoplasm. The identification of collagen in a cell-free system is complex, but a rapid assay using purified bacterial collagenase has been used with success.

Major Findings: Collagen in lung is synthesized in polysomes isolated from the endoplasmic reticulum of lung. These polysomes can be translated efficiently in a heterologous system (rabbit reticulocyte and liver) where the only components from lung are the polysomes. Synthesis of collagen in the cell-free system has been identified by molecular sieve and ion-exchange chromatography, acrylamide gel electrophoresis, sensitivity to collagenase and synthesis of hydroxyproline containing peptides. During lung development

there are significant changes in the relative proportion of lung polysomes that synthesize collagen. The percentage of collagen (relative to other lung proteins) in the cell-free system derived from the newborn rabbit is twice that in the cell-free system derived from the adult rabbit. These changes parallel the findings in control of collagen synthesis in lung explants suggesting that at least some of the control of lung collagen synthesis may be due to changes in lung collagen gene expression or due to changes in the number of lung cells that synthesize collagen.

Significance to Biomedical Research and Institute Program: At each stage of development of the other projects in this laboratory, the cell-free system can be applied to understand the control of synthesis of structural and non-structural proteins in the lung. Once the normal mechanisms are identified, comparison with human pathologic states will be made. For example, identification of an increase in a specific collagen mRNA in a certain fibrotic lung disease may help identify the primary pathology. In addition, the cell-free system is readily adaptable to studying molecular control mechanisms and identifying vulnerable steps in the process of connective tissue synthesis which may be influenced to treat specific human lung disorders.

Proposed Course to Project: As the cell-free system is refined, it will be used to quantitate the messenger RNA for specific collagen chains. A purified collagen mRNA will be used to make a reverse DNA copy to be used as a probe for quantitating the number of collagen genes during normal lung growth and in the fibrotic lung disorders. Techniques will be developed to establish cell-free systems from human lung including human lung cells maintained in culture. Investigations will concentrate on identifying the molecular mechanisms controlling normal lung growth and the fibrosis of lung following injury.

Keyword Descriptors: Lung, Protein Synthesis, Collagen, Cell-free, Messenger RNA, Translational Control, Polysomes, Initiation Factors.

Honors and Awards: None
Publications:

Collins, J.F. and Crystal, R.G. Protein Synthesis. In: R.G. Crystal (ed.). The Biochemical Basis of Pulmonary Function, M. Dekker, New York, 1975.

Collins, J.F. and Crystal, R.G. Characterization of Cell-Free Synthesis of Collagen By Lung Polysomes in a Heterologous System. J. Biol. Chem. (Submitted)

Project No. Z01 HL 02403-02 PB
1. Pulmonary Branch
2. Section on Pulmonary Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Collagen Synthesis in Cultured Lung Cells

Previous Serial Number: NHLI 109

Principal Investigators: Ronald G. Crystal, M.D.
Allan Hance, M.D.
Allen Horwitz, M.D., Ph.D.
Norton Elson, M.D.
Kathryn Bradley, M.S.
Sally McConnell-Breul, M.S.

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: The application of biochemical technology to human lung disease is limited by the complexity of the organ and the unavailability of large quantities of lung cells from patients with lung disease. There are more than 40 cell types in lung, and some of these cells are responsible for the synthesis of 5 different types of collagen chains. When the lung synthesizes abnormal amounts of collagen in response to injury, the same cells normally producing collagen may proliferate; they may synthesize relatively more collagen or cells that do not normally synthesize collagen may be "turned on" to do so. One of the primary objectives of this laboratory is to develop the technology to culture the cells from lung that are important in collagen synthesis in health and disease.

Methods: Fibroblasts and epithelial cells are cloned from primary cultures of rabbit, cat, rat or human lung cells dispersed from lung with trypsin. Macrophages are removed from lung by lavage. Methods have been developed to quantitate absolute rates of collagen and non-collagen protein synthesis in these cells utilizing intracellular measurements of specific activity of labeled amino acids and by quantitating labeled collagen with collagenase or hydroxyproline measurements. The types of collagen synthesized are determined by ion-exchange chromatography and cyanogen bromide peptide mapping techniques.

Major Findings: Fibroblasts from fetal or newborn rabbit, cat, rat and human lung actively synthesize Type I and Type III collagen. The collagen secreted into the media of these cultures is in a precursor form called pro-α chains. An epithelial cell cultured from fetal cat lung also synthesizes Type I and

Type III collagen but not as actively as the fibroblast. Pulmonary alveolar macrophages do not synthesize collagen. A method has been developed to prepare a "fingerprint" of the proteins secreted by cultured lung cells to be used as biochemical markers to identify the cell types. The "fingerprint" of the fibroblast, epithelial cell and macrophage are markedly different. Purified collagen chains isolated from the media of these cultures are being utilized to prepare antibodies against specific types of collagen. These antibodies are being used to quantitate collagen synthesis in culture, cell-free systems and biopsy specimens (by immunofluorescent methods).

Significance to Biomedical Research and Institute Program: The mechanisms by which collagen is synthesized and degraded in the lung are fundamental to the control of normal lung structure and function. In the fibrotic lung disorders these mechanisms are presumably deranged so that collagen is synthesized in either abnormal amounts and/or in abnormal regions of the lung. The establishment of diploid cell lines of the cells responsible for collagen synthesis in health and disease will allow investigations at the molecular level. This includes identification of the normal mechanisms and how these are varied following lung injury.

Proposed Course of Project: There will be continued establishment of different cell lines, the investigation of the type of collagen synthesized by each and inquiry into the mechanisms of collagen synthesis in each. One area of particular interest is the synthesis of three different collagen chains by an apparent homogeneous cell fibroblast line. Through cloning techniques, it should be possible to determine if this is in fact a single cell line. If so, investigations into the quantitative control of synthesis and degradation of each collagen type will be done. Once these techniques are established in normal animal lung cells, they will be applied to the study of human cells from normal and abnormal lungs. Immunofluorescent methods should enable the identification of collagen types in biopsy specimens from patients with different types of lung disease.

Keyword Descriptors: Lung, Collagen, Collagen Heterogeneity, Lung Cells, Macrophage, Fibroblasts, Epithelial Cell, Cell Culture.

Honors & Awards: None

Publications: Hance, A.J., Bradley K. and Crystal, R.G. Lung Collagen Heterogeneity II. Synthesis of Type I and Type III Collagen by Rabbit and Human Lung Cells in Culture. J. Clin. Invest. (Submitted)

Project No. Z01 HL 02404-03 PB
1. Pulmonary Branch
2. Section on Pulmonary Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Accumulation of Collagen in Human Lung

Previous Serial Number: NHLI 111 (c)

Principal Investigators: Ronald G. Crystal, M.D.
Morton J. Cowan, M.D.
Jack D. Fulmer, M.D.
Kathryn Bradley, M.S.
Sally McConnell-Breul, M.S.
Allan J. Hance, M.D.

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: The connective tissue of the lung is fundamental for lung structure and mechanical properties. In addition, the interstitial lung diseases, many of which result in pulmonary fibrosis, represent approximately 20% of lung diseases (other than the infectious diseases). Almost nothing is known about the composition of human lung collagen nor its synthesis and regulation. With the ultimate aim being an ability to control pulmonary fibrosis through molecular mechanisms, our laboratory is developing the technology to quantitate human lung collagen composition and synthesis.

Methods: Lung tissue is obtained from patients undergoing thoracotomy or from fetuses following therapeutic abortion. Intact collagen chains are extracted with conventional techniques. Lung collagen is synthesized in short-term explant cultures of human lung and compared and identified with the extracted collagen chains by ion-exchange chromatography, SDS and acidic acrylamide gels, sensitivity to collagenase and hydroxyproline content. Conditions have been developed to quantitate and compare the rate of collagen synthesis in biopsies from human lung.

Major Findings: Normal human lung synthesizes at least two collagen chains ($\alpha$1(I), $\alpha$2) and probably a third ($\alpha$1(III)). Fibroblasts cloned from human lung synthesize $\alpha$1(I), $\alpha$2 and $\alpha$1(III) chains. In the 16-20 week human fetal lung and in the adult human lung approximately 4% of the total amino acids incorporated into protein are incorporated into collagen. Biopsies from three patients with idiopathic pulmonary fibrosis have been examined. Interestingly,

none had increases in the percentage of protein synthesis that was collagen, suggesting that the molecular pathology in fibrotic lung disease is more than simply an increase in fibrous tissue synthesis. Methods have been developed to quantitate human lung collagen proteolysis as well as synthesis to determine the influence of rates of degradation of collagen on accumulation of collagen in these disorders.

Significance to Biomedical Research and Institute Program: In the interstitial lung disorders, the lung responds to injury by producing fibrosis resulting in functional pathology in gas exchange. Through the techniques developed in our laboratory, it should be possible to: (1) quantitate the degree of the fibrotic process (i.e., rate of collagen synthesis relative to collagen destruction) (2) develop an *in vitro* system for testing drug efficacy in these disorders.

In the emphysematous disorders there is destruction of the connective tissue comprising the lung with either absent or ineffective remodeling of the connective tissue. Of particular interest is the $\alpha$1 antitrypsin group of patients, who have an inherited disorder of the serum $\alpha$1 globulins associated with emphysema. The techniques described above can be used directly to test the influence of the serum proteins on protecting the lung from proteolysis.

Proposed Course of Project: The normal patterns of collagen synthesis and degradation will be quantitated for different age groups. Once a baseline is firmly established, it will be possible to quantitate the influence of other factors (drugs, $\alpha$1 globulins, $\alpha$2 macroglobulins) on the fibrotic process in biopsy specimens from patients with interstitial disease. It should then be possible to predict, for each patient, which drug(s) will be most efficacious.

Keyword Descriptors: Collagen, Human Lung, Pulmonary Fibrosis, Collagen Synthesis, Collagen Degradation.

Honors and Awards: None

Publications: Bradley, K., McConnell-Breul, S. and Crystal, R.G. Collagen in the Human Lung: Composition and Quantitation of Rates of Synthesis. J. Clin. Invest., 55: 543, 1975.

Crystal, R., Bradley, K., McConnell-Breul, S., Collins, J., Hance, A., and Cowan, M. Collagen in the Lung: Development of a Technology Applicable to Human Lung Disease. Chest, 67: 305, 1975.

Project No. Z01 HL 02405-02 PB
1. Pulmonary Branch
2. Section on Pulmonary Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Experimental Models of the Interstitial Lung Disorders

Previous Serial Number: NHLI 112

Principal Investigators: Ronald G. Crystal, M.D.
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Allan J. Hance, M.D.
Allen L. Horwitz, M.D., Ph.D.
Norton Elson, M.D.
William Wagner, M.S.
Jack D. Fulmer, M.D.

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: A major goal of this laboratory is to understand and eventually control the mechanisms involved in connective tissue synthesis and degradation in human lung in diseases involving these components. Because the availability of human lung biopsy material is limited, it necessitates that any biochemical techniques applied to this tissue is worked out first in animal models, thus insuring the maximum amount of information and the maximum benefit to the patient. For that reason two groups of models of fibrotic disease are being developed: (1) animal models; and (2) tissue culture models.

Methods: (1) Animal models: Silica particles, 1-5 microns in diameter are injected intratracheally in 6 week old rabbits. At intervals following exposure, the animals are killed and measurements are make of lung of collagen content, rates of collagen synthesis and the relative proportion of the proteins are being synthesized that are collagen. These are compared to litter mate paired controls. Other animal models being developed include: radiation (external) injury, hypersensitivity to inhaled antigens and chemical mediated lung injury. In all animal models, the biochemical measurements are being compared to morphologic (light and electron microscopy) and functional alterations. The latter are being studied in a newly developed in vitro plethysmograph which can be used to measure pulmonary function parameters of the parenchyma and airways.

(2) Tissue culture models: The development of methods to culture and continually passage lung cells in culture has enabled us to begin the development of

in vitro models of lung cell injury. Cultured lung cells can be interacted with other cells (macrophages, lymphocytes) or their products. They can also be studied in relation to direct irradiation or with chemical and pharmacological mediators of lung injury. Rates of connective tissue synthesis and degradation can be quantitated and cell-free methods can be applied. Morphologic alterations at the electron-microscopic level are also studied.

Major Findings: (1) Animal models - Although collagen accumulation takes place in silica exposed animals, it is a difficult model to work with because the silicotic "nodules" are discrete and inhomogeneous. Radiation induced fibrosis has been a much more satisfactory model and preliminary studies have demonstrated increased collagen density 6 weeks after 6000 R to the lung.

(2) Tissue culture models - The pulmonary alveolar macrophage can be induced to secrete collagenase, a unique and specific enzyme for mammallian collagen. Rabbit macrophages activated with BCG (in vivo) will secrete collagenase after 1-2 days in culture. Exposure of these macrophages to protein antigens derived from mycobacterium will further augment this finding. Sufficient quantities of mammallian collagenase are being purified to produce antibodies specific for it. Soon, it should be possible to have tissue culture systems involving lung cells, macrophages, lymphocytes and serum from animals, normal humans and humans with fibrotic lung disease in which the rates of synthesis and degradation of specific collagen types can be quantitated and manipulated.

Significance to Biomedical Research and Institute Program: As we learn to define the animal and tissue culture models of fibrotic lung disease, we should be able to understand the sensitive control points where we can interrupt the fibrotic process with pharmacologic agents.

Proposed Course to Project: Continued development of these models with particular emphasis on the quantitative description of the fibrotic process including the type of collagen synthesized and how this may be influenced with drugs. Other projects in our laboratory such as cell-free collagen synthesis will be applied to these models as the technology develops.

Keyword Descriptors: Lung, Fibrosis, Animal Models, Cultured Cells, Lung Injury, Macrophages, Lymphocytes, Fibrotic Lung Disease, Collagen Synthesis, Collagen Degradation.

Honors and Awards: None

Publications: None

Project No. Z01 HL 02406-02 PB
1. Pulmonary Branch
2. Section on Pulmonary Biochemistry
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Heterogeneity of Lung Collagen

Previous Serial Number: NHLI 108

Principal Investigators: Ronald G. Crystal, M.D.
Allan Hance, M.D.
Kathryn Bradley, M.S.

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: Collagen is the most abundant protein in lung, comprising 10-15% of the adult lung by dry weight. There are more than 40 cell types in lung including mesenchymal cells, epithelial cells and smooth muscle cells, all of which have been implicated in other organs to synthesize collagen. The primary objective of this project is to identify the heterogeneous collagen chains in lung.

Methods: Because the majority of collagen synthesized in lung is synthesized early in life, the collagen in the adult is mostly cross-linked and, therefore, almost impossible to extract intact. However, conditions have been developed so that 100% of the collagen synthesized in short-term explant lung cultures can be extracted and subsequently analyzed. Different lung structures (bronchial tree, blood vessels and peripheral lung) are isolated under a dissecting microscope and incubated in the presence of labeled amino acids. The collagen chains synthesized are extracted, purified and subsequently compared by cyanogen bromide peptide mapping techniques.

Antibodies to collagen chains purified by tissue culture methods are being used to relate each of the heterogeneous collagen chains to specific lung structures.

Major Findings: Prior studies demonstrated that the lung parenchyma and blood vessels synthesized $\alpha 1(I)$ and $\alpha 2$ chains (Type I collagen) while the tracheobronchial tree synthesized $\alpha 1(II)$ chains (Type II collagen). More recent studies have demonstrated $\alpha 1(III)$ chain (Type III collagen) synthesis in the parenchyma. These collagen chains have distinct primary amino acid sequences as demonstrated by cyanogen bromide peptide maps on ion exchange

columns and acrylamide gels. All evidence to date suggests that while lung collagen is markedly heterogeneous, each type collagen is identical to collagen elsewhere in the body (e.g., Type I is the same from lung, skin, bone, tendon, blood vessels; Type III is the same from lung, aorta, skin; Type II is the same from tracheobronchial tree and sternal cartilage.

Significance to Biomedical Research and Institute Program: Normal lung structure and function depends on the collagen comprising it. During the developmental process there undoubtedly are changes in the control of collagen synthesis as these structures change. An understanding of these mechanisms will help toward an understanding of the pathologic process in diseased lung.

The fibrotic lung diseases have many primary stimuli including inhalation of toxic materials, hypersensitivity states, radiation injury and associated with systemic disorders such as scleroderma. It is likely that these disorders are associated with injury to different lung cells, suggesting the possibility that there may be a heterogeneity in the types of collagen synthesized in these different diseases. It may be possible to classify the interstitial lung diseases on the basis of the types of collagen synthesized in a similar fashion to the lipoprotein disorders and certain hemoglobin disorders.

The heterogeneity of lung collagen may also be a crucially important determinant of lung mechanical properties in the normal development of lung.

Proposed Course of Project: The $\alpha 1(I)$, $\alpha 2$, $\alpha 1(II)$ and $\alpha 1(III)$ collagen chains from lung have now been isolated, purified and mapped. It is known that there is greater than 70 sq. meters of basement membrane ($\alpha 1(IV)$ collagen chains) in lung; the next step will be to isolate and purify these chains. Once that is accomplished, it should be possible to quantitate the rate of synthesis and degradation of each lung collagen type in normal lung development and in human lung disease.

Keyword Descriptors: Lung, Collagen, Collagen Heterogeneity, Peptide Mapping, Collagen Synthesis, Collagen Degradation, Tracheobronchial Tree, Blood Vessels, Lung Parenchyma.

Honors and Awards: None

Publications:

Hance, A.J. and Crystal, R.G. Collagen. In: R.G. Crystal (Ed.). The Biochemical Basis of Pulmonary Function, M. Dekker, New York, 1975 (in Press).

Project No. Z01 02407-01 PB
1. Pulmonary Branch
2. Section on Pulmonary Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies of Patients with Fibrotic Lung Disease

Previous Serial Number: None

Principal Investigators: Ronald G. Crystal, M.D.
Jack D. Fulmer, M.D.
Norton Elson, M.D.

Other Investigators: None

Cooperating Units: Bruce Line, M.D., Clinical Center, Nuclear Medicine
Herbert Reynolds, M.D., Allergy and Infectious Diseases, Laboratory of Clinical Investigation
Irving J. Selikoff, M.D., Mount Sinai School of Medicine, University of New York
Frederick J. Wenzel, M.D., Marshfield Clinic, Marshfield, Wisconsin
William Roberts, M.D., Heart Institute, Intramural Research - OD
Victor Ferrans, M.D., Heart Institute, Intramural Research - OD
Ralph Dolin, M.D., Allergy and Infectious Diseases Laboratory of Clinical Investigation

Project Description:

Objectives: The fibrotic lung disorders represent 15-20% of the non-infectious disorders of the lung. The mean survival of these patients is 45 months from the onset of symptoms. Although approximately 5-10% of these patients respond to corticosteroids, there is essentially no treatment for the remainder. The natural history, etiology and pathogenesis of "idiopathic" fibrotic lung disease is poorly studied. The Pulmonary Branch, NHLI, has undertaken a detailed study of these patients with several major objectives:

(1) To determine the etiology of idiopathic fibrotic lung disease.
(2) To follow patients longitudinally to determine (a) the natural history of this disorder and (b) which pulmonary function parameters are most sensitive to the disease process.
(3) To correlate pulmonary alveolar constituents (fluid and pulmonary alveolar macrophages) with fibrotic disease.
(4) To correlate radioisotopic monitors of lung function (ventilation, perfusion and gallium scans) with the disease process.

(5) To correlate lung pathogenic alterations with biochemical and functional changes.
(6) To study the pharmacologic therapy of this disease process.

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, roenteographic and EKG studies, serologic studies aimed at immune processes; pulmonary function studies including lung volumes, flow rates, diffusing capacity, flow-volume curves, closing volume, closing capacity, body plethysmograph functional residual volume and lung resistance at all lung volumes, static and dynamic pressure-volume curves, maximum flow static recoil curves, iso-volume pressure-flow curves, ventilatory and arterial blood gas studies at rest and exercise. Lung lavage for macrophage function and morphology, secreted proteins of macrophages and constituents of alveolar wash fluid. Technetium vascular scans, $^{133}Xe$ ventilation scans and $^{85}$gallium scans. When indicated, lung biopsy is done either through the fiberoptic bronchscope or via open thoracotomy. Tissue is studied by light microscopy, electron microscopy, culture (routine, fungal, mycobacteria and virus), collagen synthesis and degradation (see Project Report No. Z01 HL 02401-03 PB), electron probe analysis for mineral content and tissue culture (see Project Report No. Z01 HL 02403-02 PB). Selected patients are then entered into a drug treatment protocol where all patients are treated with corticosteroids (standard therapy) and 50% of the patients are treated (in addition) with azothioprine (in a double blind fashion).

Major Findings: Preliminary studies suggest:
(1) Obstruction to airflow is a significant part of the pathology of this disorder.
(2) Classic immune mechanisms (at least as defined by serologic parameters) are not frequent occurrences.
(3) Rare patients can be classified as having environmental etiologies (defined by electron probe analyses of biopsies).
(4) The biochemical pathology is much more than simply an increase in connective tissue synthesis.
(5) Ventilatory and arterial blood gas parameters with exercise are probably the most sensitive monitors of the disease process.
(6) Patients with this disorder have marked abnormalities in ventilation-perfusion mismatching.

Significance to Biomedical Research and Institute Program: These disorders are almost uniformly fatal and affect a significant proportion of the population. Up to this time, there has been no information on pathogenesis and there is essentially no cure. By combining studies on patients with these disorders with our large basic research program in the control of connective tissue accumulation in lung, we can expect to make major inroads into understanding and eventually curing these disorders.

Proposed Course to Project: Studies as outlined will be continued. As methods are developed in the basic laboratory, they will be applied to study the biopsy specimens from human lung. Particularly important are the studies in lung explants and tissue culture where the manipulation and control of lung

collagen synthesis and degradation can be studied with pharmacologic agents. As results with pharmacologic agents become promising, they will be studied in patients when applicable.

Keyword Descriptors: Fibrotic Lung Disease, Lung Collagen, Lung Fibrosis, Pulmonary Function Testing, Corticosteroids, Azothioprine, Macrophage.

Honors and Awards: None

Publications:

Fulmer, J.D. and Crystal, R.G. The Biochemical Basis of Pulmonary Function. In: R.G. Crystal (Ed.): The Biochemical Basis of Pulmonary Function. M. Dekker, New York, 1975 (in Press).

Annual Report of the
Section on Molecular Pharmacology
Pulmonary Branch
National Heart and Lung Institute
July 1, 1974 through June 30, 1975

Receptors: Dysfunctions of airway and vascular smooth muscle are most readily brought under pharmacological control by drugs acting at neuromuscular junctions where signals are transmitted to receptors in muscle membrane by chemical agents. As an aid to the design of new drugs and to understanding the selectivity of certain drugs for receptors in specific organs, the molecular events associated with receptor activation are being studied in this Section. Over the long term emphasis will be placed on α-adrenergic, serotonin and other receptors that initiate contraction, since relatively less is known about these receptors than about the β-adrenergic relaxing mechanisms mediated by cyclic AMP. Ultimately attention must be directed to the possible role of calcium release from membrane stores, activation of phospholipid turnover and stimulation of guanyl cyclase activity, all of which have been postulated to play a role in contractile events. At the moment, however, interest centers on the initiating event, the adsorption of agonist on a recognition site, and research has been directed primarily to the design of techniques for receptor isolation.

The successful synthesis of [$^{3}$H]-sulfanilic acid of high specific activity and the availability of $^{35}$S-labeled material should make it possible to use double isotope procedures to identify those components of receptors which undergo agonist-dependent changes in conformation. Sulfanilic acid was selected as a reagent because of the ease with which it can be diazotized for direct incorporation into membrane proteins or into other reagents that in turn can be reacted with membranes. Preparation of a suitable SH reagent is nearing completion and feasibility studies on the application of the technique to aortic receptors are encouraging.

Magnetic resonance and the reaction of drugs with biomolecules: Previous reports have described the search for those physical techniques most likely to be useful for exploring receptor topography and the evolution of electron spin resonance as the method of choice. The systematic study of the interaction of spin-labeled compounds with model surfaces that vary considerably in topography has continued. There has been some shift in emphasis toward studies of the fluid properties of phospholipid membranes. Changes in membrane fluidity with concomitant increases in the ease of association of proteins embedded in the membrane have been postulated to play a role in receptor response, neoplastic transformation and a variety of other membrane functions.

The most complicated surface thus far delineated by these studies is that of the biotin binding protein, avidin. ESR studies of its complexes with spin-labeled biotin analogues of variable length suggest a quadrivalent site with two pairs of clefts, the members of each pair being separated by about 16 Å. Between each pair of clefts there exist two lipophilic sites and there is a more peripheral lipophilic site in the vicinity of each cleft. Other studies of this character have shown that immobilization of aminoacridine spin labels in DNA complexes is consistent with intercalation of the amines in the helix

and that ESR can be used to study the effects of substituents on the mobility of acridines in such complexes. By inserting into cell membranes stearic acid analogues bearing spin labels at various positions along the side chain, it has been possible to measure changes in the fluid character of the phospholipid bilayer. Such studies have shown that neoplastic transformation of several cell types by a temperature sensitive mutant of Rous sarcoma virus and by SV-40 do not cause any change in membrane fluidity. Studies of the $H^+$ transporting purple membrane of Halobacterium halobium have disclosed a biological rarity, an extremely rigid membrane. Spectral evidence suggested two populations of membrane lipids, one of which is presumably tightly associated with the membrane's single protein. An interesting new feature of the ESR studies has been the use of spin trapping, i.e. the conversion of a biologically generated unstable free radical into a stable free radical sufficiently long lived to be easily studied. This procedure has been used to measure the temperature-dependent formation of free radical intermediates in the metabolism of organic halides.

Ion transport: The sodium-and potassium-dependent ATPase of cell membranes is of interest as the putative receptor for cardiac glycosides, which act extracellularly to inhibit the intracellular hydrolysis of ATP. The system is also of interest because of its general role in maintaining excitability of nerve and muscle membranes. It remains probably the most useful paradigm for theoretical studies of the relationship between lipoprotein organization in the membrane and transmembrane control of intracellular enzymic functions. Various aspects of the system are under investigation in many laboratories; the emphasis in this section is on structural studies of the system's major protein.

Current studies with a conveniently prepared purified ATPase system from kidney have shown that cyanylation of protein SH groups by 2-nitro-5-thiocyanobenzoic acid gives a stepwise inactivation, consistent with at least two populations of SH groups. Protection by the substrates, ATP and sodium, occurs with only the more slowly reactive sulfurs. Efforts to find evidence for hidden SH groups that become exposed upon phosphorylation of the system have not yet succeeded with the cyanylating reagent, although such groups can be revealed when $^{14}C$-cyanylating reagent has been synthesized and $^{13}C$-cyanylating reagent is undergoing synthesis. These compounds should make possible the determination of the relative labeling of the two protein components of ATPase. Since ligand-dependent conformational changes associated with the ion transport cycle are limited to the larger protein, future emphasis will be placed on the use of $^{13}C$ NMR to explore conformational changes in this protein and on the base catalyzed cleavage of the cyanylated protein to detect conformationally mobile loci.

Studies in lung: Studies on the mechanisms for storage and release of histamine and serotonin in the lungs of Wistar rats continue. The rapid appearance of serotonin storage mechanisms at 4-5 weeks of age was reported earlier but the physiological role of serotonin storage sites remains unclear. Release of this amine from subcellular organelles has been offered as one of several possible explanations for the rapid vasoconstriction induced by hypoxia. Uptake of circulating serotonin has been variously reported to lead to immediate metabolic degradation in epithelial cells or to partial storage in unchanged

form, depending on the laboratory reporting and on the amounts used. Further study of these questions seems desirable.

Current studies with histofluorescent techniques have shown serotonin but not histamine to be present in lung in large cells, somewhat resembling mast cells, that are clustered in the vicinity of blood vessels and intimately associated with sympathetic nerve fibers, although no synapses have been seen. Isolated slices of lung tissue will concentrate serotonin but not histamine to levels 4-5 times those in the medium when nanomolar concentrations are used. Studies of the effects of drugs on the kinetics of release from these model preparations have not yet been accomplished. In germ-free animals maintained up to 11 months, the levels of histamine but not of serotonin remain at less than 1/10 those observed in normal animals.

Surfactant isolated from rabbit lung has been examined by nuclear magnetic resonance at the $^{31}P$ frequency. Since no signals were detectable except after sonication of the preparation, the phospholipid must be highly immobilized in the intact complex. Paraquat, a drug originally investigated because of the peculiar delayed pulmonary edema that it causes in man, has some structural similarity to decamethonium. A search for possible anticholinergic effects disclosed that it has some neuromuscular blocking properties in chicks. The turnover of membrane phospholipids, particularly phosphatidic acid and phosphatidyl inositol, is often increased in tissues exposed to acetylcholine or $\alpha$-adrenergic agonists and might be expected to be diminished by paraquat. The effects of this drug on incorporation of labeled phosphate into phospholipids of rat lung slices were equivocal, amounting to small reductions in the turnover of phosphatidylglycerol and phosphatidylcholine and a modest increase in phosphatidic acid. Propranolol, which has been observed to give a small but significant reduction in paraquat toxicity in vivo, had much larger effects on phosphate incorporation as previously reported in the literature. Effects of the two drugs appeared to be additive.

Miscellaneous studies with biogenic amines: The sensitive (10-50 pg range) and specific assay methods for histamine, serotonin and catecholamines that were developed in this laboratory and used in the studies of amine turnover in lung have also made possible for the first time the accurate measurement of biogenic amines in plasma and urine. The methods have proved useful in the diagnosis and study of disorders in which amines are thought to play a role. Although histamine has been long suspected as the mediator of conditions such as cold- and exercise-induced urticaria, the present studies provide the first direct evidence that histamine is released during such conditions. Gastric ulcers associated with basophilic leukemia and gastric tumors have been shown also to be due to circulating histamine. Treatment with the $H_2$ blocker metiamide has produced remission of the ulcers in these patients.

In previously reported studies of histamine metabolism, salicylates in therapeutic doses were found to inhibit the riboside conjugation of imidazole acetic acid in man and various animal species. Studies in vitro have since shown that the salicylates inhibit directly ($K_i \sim 10^{-5}$) the conjugating enzyme. The enzyme has been isolated from liver and kidney and is unusual in that it requires both ATP and PP-ribose-P as well as magnesium. The need for this

enzyme in the body's economy is not apparent, since imidazole acetic acid itself is inactive pharmacologically and is readily excreted from the kidney without need for additional conjugation. The enzyme may be involved in nucleotide salvage pathways. There are indications in the literature that the salicylates inhibit nucleic acid synthesis in bacteria and lymphocytes during blast transformation. These observations would suggest an inhibitory action of aspirin on rapidly growing tissues, and indeed aspirin administration was found to inhibit the growth of a transplantable ascites tumor in mice and $^{14}C$-thymidine incorporation into the nucleic acid precipitable fraction of this tumor. Similar studies are underway with Lewis lung cell carcinoma, a rapidly growing solid tumor which produces death by metastasis to lung. The possible effect of aspirin on other tumors will be explored with research workers in the Cancer Institute. Our major interest in this phenomenon is that aspirin may exert its effect in inflammation by inhibition of leukocyte proliferation in inflammed tissues. It is hoped to use such models as turpentine-induced lung edema in rats for this purpose.

Project No. Z01 HL 02501-02 PB
1. Pulmonary
2. Molecular Pharmacology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Influence of Age, Infection and Drugs on Lung Amines

Previous Serial Number: NHLI-106

Principal Investigators: Dr. Michael A. Beaven
Dr. Zdenka Horakova
Mr. Richard E. Shaff

Other Investigators: Dr. David Jacobowitz
Dr. David Small

Cooperating Units: Dr. Jacobowitz is a member of the Laboratory of Clinical Science, Section on Histopharmacology, NIMH
Dr. Small is with the Division of Research Resources, Veterinary Resources Branch

Project Description:

Introduction: Our previous studies (see project report NHLI-106, 1974) have shown that in rats histamine and serotonin levels in lung are less than 0.1 µg/g at birth. The levels of histamine in lung increase progressively with age and attain levels of 7-17 µg/g by 18 months of age. Serotonin levels increased to a maximum of 4-6 µg/g by 10 weeks of age and then remained constant for the remainder of the rat's life. The studies suggested that accumulation of the amines was due to the appearance of storage mechanisms in the older animal. The two amines were resistant to agents which selectively destroyed sympathetic and serotonergic nerves, and they reside presumably in extraneuronal stores. The histamine and serotonin stores were shown to differ in several respects. Studies with monoamine oxidase inhibitors and amino acid precursors indicated that the serotonin turns over rapidly and histamine slowly in the lung. Both amines also differed in their susceptibility to reserpine and compound 48/80; serotonin but not histamine was depleted by reserpine treatment; and histamine but not serotonin was partially depleted by compound 48/80 administration. It seemed unlikely therefore that the two amines were stored in the same type of cell.

This year's study has been concerned with the histological investigation of storage sites of the amines and of the influence of infection on the levels of the amines in lung.

Histological Studies: Histological studies using the formaldehyde fluorescent techniques developed in Sweden showed that serotonin was localized exclusively in large cells surrounding the blood vessels. These cells were present along all blood vessels, and they had the characteristic morphology of mast cells. An unusual feature was the cells appeared to be intimately associated with sympathetic nerve fibers. Synapses of nerve fibers to the mast cell were not discernible, however; these cells were absent in young rats (9-day-old) as might be expected from the low levels of serotonin in lung at this age. Histological examination of histamine stores has not been made at this time.

Pharmacological Studies: Continued studies in vivo and in vitro point to further differences in the storage of histamine and serotonin in lung. Experiments with lung slices in vitro have confirmed our earlier impression that lung possesses the ability to take up serotonin but not histamine. Tritiated serotonin, for example, is rapidly taken up into lung slices by a saturable process that is temperature dependent and which is inhibited by metabolic inhibitors, such as iodoacetate, N-ethylmaleimide and cyanide. Concentration gradients of 1:3.5 to 4.5 (medium to slice ratio) were consistently obtained. The uptake was blocked by agents that are known to block the uptake of serotonin into brain slices. In contrast to serotonin, passage of tritiated histamine into lung slices was slow, and the ratio of histamine in medium to tissue did not exceed 1. These studies suggested serotonin but not histamine is taken up by a transport system into lung tissue.

Studies with Germ-Free Rats: An important aspect of this year's work has been the finding that histamine levels are very low in lung of germ-free rats. In these rats, lung histamine ranged from 0.08 $\pm$ .02 ($\pm$ SEM, n = 10) µg/g at birth to 0.87 $\pm$ 0.13 µg/g (n = 8) at 11 months of age. After removal of rats from the germ-free barrier, there is a gradual and progressive increase in histamine levels in lung. For example, after 1, 3, 7, 11, 20, 30 weeks and 11 months, the lung histamine was 1.1 $\pm$ 0.1, 2.0 $\pm$ 0.2, 2.3 $\pm$ 0.8, 4.8 $\pm$ 0.9, 7.5 $\pm$ 2.6 and 11.7 $\pm$ 5.4 µg/g, respectively.

Presently, studies in collaboration with Dr. David Small (Veterinary Resources Branch) are underway to identify the specific organisms that are responsible for the increase in lung histamine. Germ-free rats are being exposed to organisms known to infect rat lung. Histamine levels and histidine decarboxylase activity, the enzyme responsible for the synthesis of histamine in tissues, are being followed. Subsequent studies will be concerned with the mechanism by which bacteria increase lung histamine and whether antigen-induced release of histamine is increased in infected rats compared to germ-free rats, using the in vitro lung model of Webseter and collaborators.

The current findings raise two important questions. Firstly, that there appear to be differences in the storage of histamine and serotonin. It has been assumed that the two amines coexist in mast cells in rat. Secondly, histamine but not serotonin is responsive to the bacterial environment. Our results suggest that infection raises the level of histamine in lung tissue. This would presumably lead to more pronounced symptoms during allergic release

of histamine. It is possible that the role of serotonin in lung is physiological and histamine pathological. The proximity of serotonin to blood vessels suggests that this amine may be important in the regulation of blood vessels.

Keyword Descriptors: Rat lung, histamine, serotonin, germ-free rats, infection, amine-storage

Honors and Awards: None

Publications:

Webster, M.E., Newball, H.H., Oh-Ishi, S., Takahashi, H., Horakova, Z., Atkins, F.L. and Beaven, M.A.: Release of histamine and arginine esterase activity from passively sensitized human lung by ragweed antigen. Cienc. Cult. 26: 372-376, 1974.

Baxter, J.H., Beaven, M.A. and Horakova, Z.: Effects of adrenergic agents, theophylline, and other drugs on dextran edema and histamine release in rats. Biochem. Pharmacol. 23: 1211-1217, 1974.

Atkins, F.L. and Beaven, M.A.: Studies of ornithine decarboxylase and histaminase (diamine oxidase) activities in rat thymus and their relationship to the thymus lymphocyte. Biochem. Pharmacol. 24: 763-768, 1975.

Beaven, M.A. and Shaff, R.: Identification of histaminase and diamine oxidase activities in various tissues of rat by sensitive isotopic assay procedures. Biochem. Pharmacol., in press.

Project No. Z01 HL 02502-02 PB
1. Pulmonary
2. Molecular Pharmacology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Influence of Aspirin on Amine and Purine Metabolism in Tumor Cells

Previous Serial Number: NHLI-107

Principal Investigators: Dr. Michael A. Beaven
Dr. Zdenka Horakova
Dr. Maria Christina de Mello

Other Investigators: Dr. Valdemar Hial

Cooperating Units: Dr. de Mello is the recipient of a fellowship from the Brazilian National Research Council
Dr. Hial is a member of the Hypertension-Endocrine Branch, NHLI

Project Description:

Introduction: The present studies stemmed from our earlier findings that aspirin and salicylates in man and other animal species inhibit the formation of ribosyl imidazole acetic acid in vivo and in vitro (see current project report Z01 HL 00604-01 LCM). Salicylates also inhibit nucleotide synthesis and growth of aerobic and anaerobic bacteria in concentrations that can be considered pharmacological (Schwartz and Mandel, Biochem. Pharmacol. 21: 771, 1972). Similar effects on nucleotide synthesis have been noted in mammalian cell systems in studies reported by Janakidevi and Smith (J. Pharm. Pharmacol. 22: 51, 58 and 249, 1970). These findings led us to investigate the possibility that aspirin might preferentially inhibit growth of rapidly growing tissues. Aspirin in moderate doses was found to inhibit growth of a transplantable mouse ascites tumor and of a solid tumor, Lewis lung cell carcinoma. These two tumors were chosen for study because of their rapid growth and the ability to measure their volume. The production of histamine by the ascites tumor was also followed. Studies with labeled thymidine and uridine were undertaken to assess the effect of aspirin on tumor RNA and DNA synthesis.

Methods: Six-week-old $CD_{f1}$ mice were housed in isolators under controlled lighting conditions and temperature. The transplantable ascites tumor of Dunn and Potter, obtained from Mr. Sidney Yancey of the Laboratory of Chemical Pharmacology, NCI, and subsequently maintained in our laboratory, was transferred to new animals weekly. Aliquots of 0.1 ml of ascites fluid



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were diluted to 10 ml with sterile Locke's solution and 0.1 ml of this suspension was injected i.p. into each mouse. Lewis lung cell carcinoma was obtained from Dr. D. S. Zaharko, Laboratory of Chemical Pharmacology, NCI. This tumor was transplanted by means of a trocha #13 under the surface of the skin.

Aspirin or vehicle was administered by stomach tube twice daily on the day before and the days following inoculation with tumor. Body weight was measured daily. Tumor volume was measured by opening the abdomen and draining the fluid from the abdominal cavity into a glass tube. Thymidine and uridine incorporation into nucleic acids was determined by injection of $C^{14}$-labeled nucleotides directly into the intraperitoneal cavity and removal of samples of tumor at different intervals after the injection of label. The nucleic acids were isolated by precipitation with trichloroacetic acid. Nucleotide incorporation was also studied in vitro in cultures of the ascites tumor cells. Histamine and serotonin were assayed by enzymatic derivative isotope techniques described in previous project reports.

Results: Studies in vivo: Aspirin treatment inhibited the increase in body weight which accompanied development of the ascites tumor. The differences in body weight between aspirin and vehicle-treated groups of mice were highly significant ($p < 0.01$) in four separate experiments. In mice that were not inoculated with ascites tumor, aspirin treatment did not alter body weight. Tumor volume and a number of tumor cells were reduced significantly (approximately 40%, $p < 0.001$) in all experiments. The size and number of cells per unit volume of ascites tumor remained unchanged. Although the concentration of histamine in the tumor was unchanged, the total amount of histamine excreted in urine was reduced by 45% in mice treated with aspirin. This reduction would be expected from the reduced tumor size.

$^{14}C$-Thymidine incorporation into the TCA insoluble fraction was reduced in mice treated with aspirin, although the difference was not significant due to a large variation in individual values for both vehicle-treated and aspirin-treated mice. $^{14}C$-Uridine incorporation was unaffected by aspirin treatment.

Studies with Ascites Tumor Cells in vitro: The uptake and incorporation of $^{14}C$-thymidine and uridine into TCA precipitable RNA and DNA is being studied in vitro in suspensions of the ascites tumor cells. In Locke's solution, the incorporation of the labeled nucleotides into nucleic acids is rapid when the cells are initially removed from the animal but diminishes as the incubation continues and increases after 2 hours. The rate of incorporation of labeled thymidine is reduced significantly in the presence of aspirin in concentrations of 1-3 mM. These findings are preliminary, and currently studies are being carried out with more complete media (Eagle's) in which the rate of nucleotide incorporation into nucleic acid does not diminish with time.

Conclusions: These studies indicate a possible effect of aspirin on tumor growth. The possibility that aspirin acts on growth of other tumors will be explored with research workers in the National Cancer Institute. Our

major interest in this phenomenon is whether aspirin exerts similar effects in inflammation and whether the major effect of aspirin is in reduction of monocyte proliferation in the inflammed tissue. It is hoped to use such models as the rat paw and terpentine-induced lung edema in rat. Aspirin is known to be particularly effective in chronic inflammatory conditions, such as arthritis, where macrophage activity may be an important component.

Keyword Descriptors: Aspirin, salicylates, inhibition of tumor growth, ascites tumor, Lewis lung cell carcinoma, aspirin and nucleic acid synthesis.

Honors and Awards: None

Publications:

Beaven, M.A., Horakova, Z. and Keiser, H.: Inhibition by aspirin of ribose conjugation in the metabolism of histamine. Eur. J. Pharmacol. 29: 138-146, 1974.

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Project No. Z01 HL 02503-03 PB
1. Pulmonary
2. Molecular Pharmacology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Disordersof Amine Metabolism in Human Disease

Previous Serial Numbers: NHLI-263(c), NHLI-94(c) and NHLI-95(c)

Principal Investigators: Dr. Zdenka Horakova
Dr. Michael A. Beaven

Other Investigators: Allen Kaplan, M.D.
Morton Grossman, M.D.
Harry Keiser, M.D.
George P. Canellos, M.D.
Richard E. Shaff

Cooperating Units: Dr. Kaplan is with the Laboratory of Clinical Invesigation, NIAID
Dr. Grossman is with the Veterans Administration Hospital, Center of Digestive Diseases, Los Angeles, Calif.
Dr. Keiser is Deputy Chief of the Hypertension-Endocrine Branch and Head of the Section on Experimental Therapeutics, HE, NHLI
Dr. Canellos is with the Medicine Branch, NCI

Project Description:

The development of sensitive enzymatic isotope assay procedures for the measurement of biogenic amines now makes it possible to assay the amines in plasma and urine. These assays are specific and 100-200 times more sensitive than the older fluorometric procedures. Over the past few years (see project reports 1972-1974), isotopic assays for histamine, serotonin and most recently the catecholamines have been established in this laboratory. These have been used to study a variety of diseases associated with defects of amine storage and metabolism and include immediate hypersensitivity reactions (studies conducted with Dr. Allen Kaplan), diseases of the gastrointestinal tract in which histamine or serotonin may be involved (Dr. Morton Grossman) and amine-producing tumors, such as basophilic leukemia carcinoid and medullary thyroid carcinoma (Drs. Canellos and Keiser). In addition to these studies, urine, plasma and tissue samples are received (about 200 per year) from outside hospitals with requests for assays of the amines or of the various enzymes associated with amine metabolism. The principal assays were shown in a table in last year's report.



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Assay Procedures: The assays for the biogenic amines take advantage of the fact that the major route of metabolism of the amines is methylation, either of hydroxyl or the alkylamine groups, and that radioactive metabolites of the amines can be formed by incubation of the sample with radioactive methyl donor, S-adenosyl-L-methionine ($^{3}H$ or $^{14}C$-methyl label), and the appropriate methyltransferase enzyme. These enzymes are readily prepared by ammonium sulfate fractionation of extracts of pineal, brain, or liver. The radioactive metabolite is then separated from the excess S-adenosylmethionine and assayed for radioactivity. When the enzymes are specific, as in the case of histamine-N-methyltransferase and hydroxyindole-O-methyltransferase, relatively simple extraction procedures can be used. When the enzymes are less specific, as in the case of catechol-O-methyltransferase, additional isolation steps are required. The techniques for separation of the enzymes and the design of this type of assay stem largely from the work of Dr. Axelrod.

Tritium release assays have been developed in this laboratory for histaminase, tryptophan hydroxylase and, recently, dopamine-β-hydroxylase. These assays utilize substrates with tritium label attached to points where proton exchange is expected to occur during the enzymatic reaction. The released tritium is measured in water by sublimation of water directly from the incubation mixture. The assays are simple, precise and are sufficiently sensitive to detect the conversion of $10^{-15}$ moles of substrate. The first assay to be developed in this laboratory, that of histaminase, has been used extensively in investigations of medullary carcinoma of the thyroid and other disorders (see earlier project reports).

Studies in Patients with Cold- and Exercise-Induced Urticaria: A total of 8 patients with cold- or cholinergic-induced urticaria have been studied. In all patients, exposure of one arm to cold or vibration resulted in release of histamine into plasma of blood reaching the brachial vein. In most patients, plasma histamine levels increased from < 1 to 8-13 ng/ml. In one patient, plasma histamine levels rose to over 30 ng/ml in the brachial vein blood and also rose (to 5 ng/ml) in the systemic circulation (blood from the opposite arm). This patient experienced mild shock and dizziness. In all patients, the symptoms ameliorated as the histamine levels declined. No histamine could be detected in plasma in control patients subjected to the same treatment. The release of histamine in a cold-induced urticaria appeared to be mediated by IgE antibody, since transfer of an IgE fraction from one of these patients to a normal subject resulted in the appearance of symptoms and release of histamine in this subject when exposed to cold.

A ninth patient (an ex-professional wrestler) with exercise-induced urticaria was unusual in that his symptoms were not relieved by the customary administration of antihistaminics. This patient showed no release of histamine or bradykinin but was found to have elevated serotonin plasma levels. Administration of methysergide aborted his attacks much to his relief, since he was troubled by his attacks during his sexual activities. This problem had been of particular concern to him and his wife during the past year.

Studies in Patients with Histamine-Producing Tumors Associated with Severe Gastric and Peptic Ulcers: One patient who has a gastric carcinoma and elevated plasma histamine levels had been located (patient of Dr. Grossman). The patient has severe gastric and peptic lesions. Histamine levels in plasma of this patient have been consistently 5-7 ng/ml. This was the first patient in which we have detected histamine in plasma under normal resting conditions. Histamine excretion in his urine was also elevated, 300-400 µg/24 hr, compared to a urine excretion of 17 $\pm$ 14 (n = 36, range < 5-51 µg/24 hr) in normal subjects and patients with miscellaneous diseases. Since studies with labeled histamine in humans have shown that only 1-3% of the histamine entering the blood stream is excreted unchanged in urine, this elevated urine excretion could represent a total excretion of over 10 mg of histamine per day. Currently, the patient is being treated with metiamide, one of the new $H_2$ histamine blocking drugs.

In addition to the above patient, two other patients (of Dr. Canellos, NCI) with Philadelphia chromosome chronic granulocytic leukemia developed an accelerated phase of the disease with high white blood cell count, high histamine levels in blood (10.5 and 11.5 µg/ml, respectively, compared to 0.08 $\pm$ 0.03 [n = 17] µg/ml in whole blood of normal subjects) and high urine excretion of histamine, 326 and 1011 µg/24 hr. One patient had symptoms of hyperhistaminemia associated with wheezing, urticaria and pruritis. The second had peptic ulcers. The second patient also had elevated histamine levels in plasma (up to 40 ng/ml). Treatment with metiamide resulted in improvement in both patients and a reduction in gastric secretion in the second patient.

Studies in other Diseases with High Histamine or Serotonin Excretion: To facilitate these studies, the isotopic serotonin assay has been adapted for measurement of serotonin in urine (see Project Report Z01 HL 02504-01 PB). To date elevated histamine excretion has been detected in 3 basophilic leukemics average 861, range 323-1400 µg/24 hr; 5 mastocytosis, average 121 $\pm$ 74, range 30-231 µg/24 hr; and one patient with urticaria pigmentosa. Apart from the patients with basophilic leukemia, histamine levels were not elevated in blood. Raised histamine or serotonin has not been detected in a variety of other diseases tested to date.

These studies will be continued using the isotopic assays and will be used to evaluate compounds that are known to antagonize or alter the metabolism of histamine and serotonin.

Keyword Descriptors: Enzymatic isotope derivative assays, histamine, serotonin, catecholamines, gastric carcinoma, immediate hypersensitivity reactions, basophilic leukemia, hyperhistaminemia

Honors and Awards: None

Publications:

Beaven, M.A.: Assay of Neurotransmitters and Drugs by Isotope Dilution Derivatization Techniques. In Iverson, I.L. and Snyder, S. (Eds.): Handbook of Neuropharmacology, Vol. I. New York, Plenum Press, in press.

Beaven, M.A. and Horakova, Z.: Assay of Histamine and Histamine Metabolizing Enzymes by Enzymatic Isotope Dilution Analysis. In Rocha e Silva, M. (Ed.): Handbook of Experimental Pharmacology. Berlin, Springer-Verlag, in press.

Kaplan, A.P., Gray, L., Horakova, Z., Shaff, R.E. and Beaven, M.A.: Mediator release in cold urticaria and cholinergic urticaria. J. Lab. Clin. Invest., in press.

Beaven, M.A., Baylin, S.B., Marshall, J.R. and Sjoerdsma, A.: Rise of plasma histaminase activity during early human pregnancy. J. Clin. Endocrinol., in press.

Baylin, S.B., Beaven, M.A. and Horakova, Z.: Increased appetite and weight gain after aminoguanidine treatment. Experientia, in press.

Project No. Z01 HL 02504-01 PB
1. Pulmonary
2. Molecular Pharmacology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Sensitive Assay for Serotonin in Tissues

Previous Serial Number: None

Principal Investigator: Mr. Richard E. Shaff

Other Investigator: Dr. Michael A. Beaven

Cooperating Units: None

Project Description:

The purpose of the present work was to develop an enzymatic double isotope assay for serotonin in which $^{3}$H-serotonin is added as an internal standard and $^{14}$C-methyl labeled S-adenosylmethionine (SAMe-$^{14}$C) is used as a methyl donor. A single isotope assay in which $^{3}$H-methyl labeled S-adenosylmethionine (SAMe-$^{3}$H) is used as a methyl donor has been previously described (Saavedra et al., J. Pharmacol. Exp. Ther. 186: 508, 1973). In our experience, there is considerable variation in recovery of serotonin from tissue to tissue, and there is a need to monitor recovery with an internal standard. It was decided to use a side chain label β-$^{3}$H-serotonin for this purpose, since the generally labeled $^{3}$H-serotonin that is commercially available is too unstable.

1) Serotonin (β-$^{3}$H-serotonin ) + Acetyl-CoA $\xrightarrow[\text{acetylating enzyme}]{\text{Rat liver}}$ N-acetylserotonin ($^{3}$H)

2) N-Acetylserotonin ($^{3}$H) + SAMe-$^{14}$C $\xrightarrow[\text{HIOMT}]{\text{Pineal}}$ $^{14}$C-melatonin ($^{3}$H)

In the first step, the sample is incubated with a tracer amount of β-$^{3}$H-serotonin (side chain label), acetyl-CoA and an acetyltransferase system prepared from rat liver (Reaction 1). After a 30-min incubation, the mixture is further incubated with SAMe-$^{14}$C and hydroxyindole-O-methyltransferase (HIOMT) to form $^{14}$C-labeled melatonin (Reaction 2). Unlabeled melatonin is added as carrier, and the melatonin is then separated from excess SAMe-$^{14}$C by extraction into toluene. The first reaction is necessary because serotonin, unlike N-acetylserotonin, is a poor substrate for HIOMT. A standard curve is prepared by taking known amounts of serotonin through the procedure. The assay is specific and sufficiently sensitive to measure 1-2 pmoles (20-40 pg) of serotonin.



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The assay has been used extensively in our studies of lung amines (see Project Report No. Z01 HL 02501-02 PB) and in studies with human subjects (see Project Report No. Z01 HL 02503-03 PB). The assay has also been modified to measure serotonin and N-acetylserotonin in urine specimens. For the latter, the urine is split into two samples. The first sample is assayed by the normal procedure to obtain values for serotonin and N-acetylserotonin; the other specimen is assayed by omitting the first reaction. This measures the amount of N-acetylserotonin present. These assays have shown that significant amounts of both serotonin and N-acetylserotonin are excreted in normal human urine.

Surveys of various rat tissues have shown that lungs contain the highest level of serotonin in the body (4-6 μg/g). Intestine, whole blood and spleen also contain relatively high levels (1-2 μg/g) of amine as has been observed in studies with the fluorometric assays of serotonin. Unlike the fluorometric assays, the isotope procedure detected no serotonin in the heart.

The use of side chain labeled β-$^{3}$H-serotonin has allowed us to study the kinetics of the two reactions outlined above. The second reaction is quantitative and goes to completion, whereas the first reaction proceeds to about 30% completion. Addition of N-acetylserotonin, the product of the reaction, further reduces the rate of conversion of serotonin to N-acetylserotonin in a competitive manner, but the reaction proceeds to the same equilibrium point irrespective of the amount of serotonin originally present. We have attempted to overcome this inhibition by coupling both reactions, i.e. adding both enzymes and cofactors in the same incubation, but this results in higher values for the blanks.

Keyword Descriptors: Serotonin, N-acetylserotonin, urine excretion, enzymatic isotope derivative assay.

Honors and Awards: None

Publications: None

Project No. Z01 HL 02505-01 PB
1. Pulmonary
2. Molecular Pharmacology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Interaction of Adrenochrome with Red Cell Ghost Membranes

Previous Serial Number: None

Principal Investigator: Dr. David B. Millar

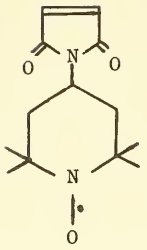
Other Investigator: Dr. Colin F. Chignell

Cooperating Unit: Dr. Millar is Chief of the Laboratory of Physical Biochemistry, Environmental Sciences Department, Naval Medical Research Institute, Bethesda, Md.

Project Description:

Certain musculoskeletal traumatic injuries have been observed to lead to a hemolytic process known as chronic anemia of trauma. Although the precise mechanism of hemolysis is at present unknown, it has been suggested that adrenochrome and perhaps additional other serum factors may be involved (Valeri et al., J. Med. 3: 20, 1972). If adrenochrome is indeed the causative agent, then it seems reasonable to postulate the interaction of this metabolite with the erythrocyte membrane may ultimately lead to destruction of the membrane and lysis of the cell. We have therefore examined the interaction of adrenochrome semicarbazide (ADCS) with human red cell ghost membranes by means of several spectroscopic techniques including circular dichroism, fluorescence spectroscopy, and electron spin resonance.

Human red cell ghosts were prepared from outdated blood bank blood by the method of Dodge et al. (Arch. Biochem. Biophys. 100: 119, 1963). Ghost preparations were repeatedly washed and dialyzed until hemoglobin was spectrophotometrically undetectable. The ghosts were sonicated in an ice bath, then stored at $4^{\circ}$ and used within 2 days of sonication. Fluorescence titrations were performed at $25^{\circ}$ in an Aminco-Bowman fluorometer. Circular dichroism measurements were made at $27^{\circ}$ in a Cary 60 spectropolarimeter equipped with a 6001 attachment. Electron spin resonance (ESR) measurements were made with a Varian E-4 spectrometer. The erythrocyte ghost membranes were reacted with spin labels I or II ($10^{-4}$ M) at $4^{\circ}$ overnight. Unreacted spin label was removed by repeated centrifugation and resuspension in buffer. Erythrocyte ghost membranes were labeled with stearic acid probe III by the addition of a methanolic solution ($2 \times 10^{-3}$ M) to the membranes.



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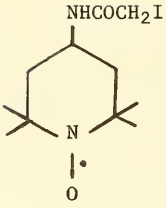
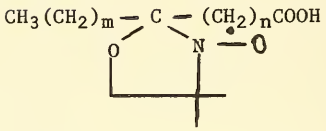


II



III

In the presence of sonicated ghost membranes, 1-anilino-8-naphthalene sulfonic acid (ANS) showed a strong symmetrical fluorescence emission band with an apparent maximum at 485 nm when activated at either 265 nm or 380 nm. The addition of ADCS to solutions containing the membranes and ANS resulted in quenching of the fluorescence of the probe. Analysis of the quenching data by means of the Stern-Vollmer relationship indicated that the quenching of the ANS fluorescence by ADCS was probably due to a collision of the adrenochrome derivative with the membrane bound probe. In contrast to the intact ghost membranes in which approximately 40% of the proteins are present in the α-helical structure (Lenard and Singer), the proteins of the sonicated ghost membranes existed to the extent of about 90% in the α-helical form. ADCS ($2 \times 10^{-5}$ M) had no detectable effect on the tertiary structure of the sonicated membranes as measured by circular dichroism.

ADCS also had no effect on the ESR spectrum of sonicated ghosts labeled with spin labels I or II. These observations again suggest that ADCS has no effect on the conformation of ghost membrane proteins. ADCS also did not change the electron spin resonance spectrum of stearic acid label III (12,3) incorporated into the sonicated ghost membrane. This would indicate that ADCS does not alter the organization of the lipids of ghost membranes.

These results suggest that while adrenochrome does bind to human red cell ghost membranes it is unlikely to be the sole causative agent responsible for post trauma hemolysis. No further studies on the interaction of ADCS with ghost membranes are contemplated at this time.

Keyword Descriptors: Musculoskeletal traumatic injury, hemolysis, adrenochrome, fluorescence, electron spin resonance, spin labels, circular dichroism.

Honors and Awards: None

Publications:

Millar, D.B. and Chignell, C.F.: Partial characterization of the 1-anilino-8-naphthalene sulfonate-adrenochrome semicarbazide interaction site in erythrocyte ghost membrane fragments. J. Biophys., in press.



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Chignell, C.F.: A topographical study of the active site of erythrocyte carbonic anhydrase by means of spin labeled drugs. J. Pharm. Sci. 64: 512-515, 1975.

Chignell, C.F.: The Fluorescence of Drug-Protein Interactions. In Chen, R.F. and Edelhoch, H. (Eds.): Concepts in Biochemical Fluorescence Spectroscopy. New York, Marcel Dekker, Inc., in press.

Chignell, C.F.: Fluorescence Spectroscopy as a Tool for Monitoring Drug-Albumin Interactions. In Morselli, P.L., Garattini, S. and Cohen, S.N. (Eds.): Drug Interactions. New York, Raven Press, 1974, pp. 111-122.

Chignell, C.F.: Protein binding and drug action. Ann. Rep. Med. Chem. 9: 280-289, 1974.

Chignell, C.F.: Ligand Binding to Plasma Albumin. In Sober, H.A. (Ed.): Handbook of Biochemistry. Cleveland, Ohio, The Chemical Rubber Co., in press.

Chignell, C.F.: Protein Binding. In Garrett, E.R. and Hirtz, J. (Eds.): Methods in Drug Metabolism Research. New York, Marcel Dekker, Inc., in press.

Project No. Z01 HL 02506-02 PB
1. Pulmonary
2. Molecular Pharmacology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Spin Trapping Studies of the Microsomal Metabolism of $CCl_3Br$

Previous Serial Number: NHLI-102

Principal Investigator: Dr. Victorio Wee

Other Investigator: Dr. Colin F. Chignell

Cooperating Unit: Dr. Wee is a Visiting Fellow of the NHLI

Project Description:

It has been postulated that $CCl_4$ is metabolized by the liver to give free radical species which are thought, in turn, to be responsible for the hepatotoxicity of this compound. The high chemical reactivity of free radical intermediates makes their detection at room temperature very difficult. In previous studies, we have attempted to detect the formation of free radicals during the metabolism of $CCl_3Br$ by rat liver microsomes with the aid of spin labeled analogues of stearic acid and choline. One of the problems with this approach is that, although we were able to observe chemical destruction of these nitroxides during the metabolism of $CCl_3Br$ by rat liver microsomes, it was difficult to establish that their disappearance was due to a reaction with free radical intermediates. Spin trapping is a technique in which short-lived free radicals react with a nonparamagnetic compound to produce a stable free radical (Janzen, Accounts Chem. Res. 4: 31, 1971). We therefore decided to attempt to trap free radical intermediates produced during the metabolism of $CCl_3Br$ with the aid of phenyl-t-butyl nitrone (I) and 2-methyl-2-nitrosopropane (II). It has been shown that when these compounds react with chemically generated free radicals they produce the relatively stable nitroxide analogues III and IV, respectively.

Rat liver microsomes were prepared from male Sprague-Dawley rats that had been pretreated with phenobarbital (80 mg/kg) for 5-7 days. Electron spin resonance (ESR) measurements were made with a Varian E-4 spectrometer equipped with a variable temperature accessory.

When trapping agent I (.01 M) was incubated with rat liver microsomes in the presence of $CCl_3Br$, TPNH, and a TPNH regenerating system, a free radical signal consisting of a triplet ($a_1$ = 15 G) in which each of the major hyperfine lines was split into a doublet ($a_2$ = 4.5 G). This triplet could only be



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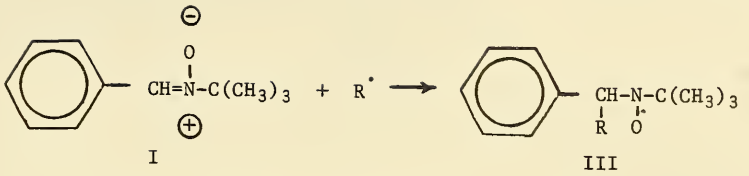
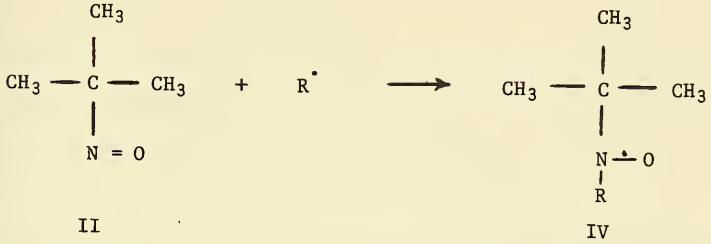


I III



II IV

observed when the sample was incubated at 37°. If the temperature of the sample was decreased to 25°, the ESR signal disappeared and could be regenerated by warming the sample again for 37°. No signal was observed in the absence of either $CCl_3Br$ or TPNH with its accompanying regenerating system.

Experiments with spin trap II were less successful. One of the problems with this label is that under the influence of light it spontaneously reacts with solvent molecules to produce stable free radical species. It was impossible to adjust experimental conditions so that these free radical intermediates were absent. The presence of a solvent generated free radical species from spin label II made experiments in the presence of microsomes and $CCl_3Br$ difficult to interpret. No further studies were therefore carried out on this compound.

Halogenated hydrocarbons are extensively used as solvents in many industrial processes. Therefore it becomes extremely important to understand the mechanism whereby these compounds cause toxicity.

Studies with spin trap I will be continued and extended. Attempts will be made to isolate and characterize the stable free radical species formed in the presence of microsomes, $CCl_3Br$ and TPNH.

Keyword Descriptors: Spin traps, electron spin resonance, carbon tetrachloride, bromotrichloromethane, phenyl-t-butyl nitrone, and 2-methyl-2-nitrosopropane.

Honors and Awards: None

Publications:

Chignell, C.F. and Starkweather, D.K.: A spin label study of human erythrocyte ghost membranes damaged by methyl phenyldiazene carboxylate. Life Sci. 14: 641-652, 1974.

Project No. Z01 HL 02507-02 PB
1. Pulmonary
2. Molecular Pharmacology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Acetylcholinesterase from Torpedo californica

Previous Serial Number: NHLI-101

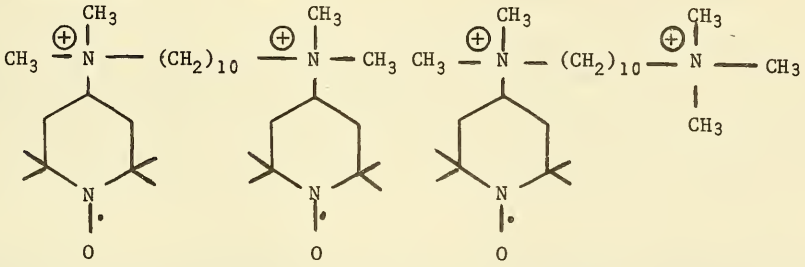
Principal Investigator: Dr. Colin F. Chignell

Other Investigators: Dr. Victorio Wee
Dr. Birandra K. Sinha
Dr. Palmer W. Taylor, Jr.

Cooperating Units: Dr. Wee is a Visiting Fellow of NHLI
Dr. Sinha held a Visiting Fellowship, NHLI, and is now a Guest Worker supported by the Microbiological Associates, Bethesda, Md.
Dr. Taylor is Associate Professor of Pharmacology at the University of California, San Diego

Project Description:

It is the aim of this study to examine the topography of acetylcholinesterase and the acetylcholine receptor protein isolated from Torpedo with the aid of spin-labeled inhibitor ligands (I, II).



I II

Acetylcholinesterase was isolated from the electroplax of Torpedo californica by previously published procedures (Mol. Pharmacol. 10: 78, ibid 10: 93, 1974). The cholinergic receptor was isolated from the same tissue using the procedure of Schmidt and Raftery (Biochemistry 12: 852, 1973). The spin-labeled ligands (I, II) were prepared by previously reported procedures



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(NHLI-104). Electron spin resonance (ESR) measurements were made with a Varian E-4 spectrometer equipped with a quartz aqueous sample cell.

Previous studies have shown that spin labels I and II were more effective inhibitors of Torpedo acetylcholinesterase than decamethonium itself. The ESR spectrum of a dilute aqueous solution of spin label I consisted of three sharp lines. However, the measurable spin concentration of I was only one-third of that which would be expected from an equimolar solution of noninteracting spin label. This suggests that the nitroxide groups at each end of the molecule come close enough to permit an exchange of spin states. By increasing the temperature, a more rapid exchange rate occurred and a five-line ESR spectrum was observed. The spin concentration calculated from this spectrum closely approximated that of a noninteracting label. When spin label I bound to Torpedo acetylcholinesterase, its ESR spectrum was broad and highly asymmetric. A splitting of 69 G between high and low field extrema suggested that the drug was strongly immobilized. The spin concentration of I bound to acetylcholinesterase was the same as that of a noninteracting nitroxide which suggested that the distance between the nitroxide groups was large enough to prevent spin exchange from taking place. The ESR spectrum of spin label I and Torpedo acetylcholinesterase appeared to have a single bound component which suggests that both ends of the molecule are immobilized to the same extent. The ESR spectrum of spin label II bound to acetylcholinesterase was similar to that described for spin label I.

Attempts to demonstrate any interaction between spin labels I and II and the acetylcholine receptor have been so far unsuccessful. The main problem with these kinds of measurements is the low concentration of receptor which we are currently able to obtain. However, Dr. Taylor has developed a new technique involving partitioning of receptor rich membranes between two phases which it is hoped will enable him to isolate the purified acetylcholine receptor in greater quantities. This should be possible to study the interaction of labels I and II with the receptor.

Acetylcholinesterase provides a very useful model system for examining the molecular basis for drug-receptor interactions. In addition, this enzyme is functionally important, since it is responsible for the destruction of acetylcholinesterase that diffuses away from the receptor surface. The possibility of isolating the acetylcholine receptor in sufficiently high quantities will make it feasible to study directly on a molecular level the interaction between a receptor and its agonist.

The electron spin resonance studies with labels I and II will be extended to chemically modified acetylcholinesterase in which the catalytic serine has been sulfonylated or phosphorylated. Studies with these modified enzymes and spin label II should be of interest, since it may be possible to detect whether the nitroxide is binding to the catalytic site or to the peripheral anionic site. Spin label studies of complexes between spin labels I and II and acetylcholinesterase in the presence of ligands such as propidium diiodide which are known to bind to the peripheral anionic site should be also of interest. With the possibility of obtaining pure membrane fragments containing

high amounts of the acetylcholine receptor, it should be possible to carry out spin-labeled studies of the receptor molecule as it exists in the membrane. Studies of the solubilized receptor should also make it possible to compare the conformation of the acetylcholine receptor in the membrane and as it is isolated in solution.

Keyword Descriptors: Spin labels, electron spin resonance, acetylcholinesterase, decamethonium, acetylcholine receptor.

Honors and Awards: None

Publications: Sinha, B.K. and Chignell, C.F.: The synthesis and pharmacology of some spin-labeled analogs of biotin, hexamethonium, decamethonium, dichlorisoproterenol and propranolol. J. Med. Chem., in press.

Project No. Z01 HL 02508-01 PB
1. Pulmonary
2. Molecular Pharmacology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Membrane Fluidity in Contact Inhibited and Transformed Cells

Previous Serial Number: None

Principal Investigator: Dr. Colin F. Chignell

Other Investigator: Dr. John Bader

Cooperating Unit: Dr. Bader is head of the Section on Cell Growth Regulation in the Chemistry Branch, NCI

Project Description:

Barnett and co-workers (Proc. Natl. Acad. Sci. U.S.A. 71: 1992, 1974) have carried out spin label studies of the membranes from contact inhibited mouse embryo fibroblast 3T3 cells and 3T3 cells transformed by oncogenic RNA and DNA viruses and by a chemical carcinogen. Their experiments suggested that the membranes of transformed cells have a higher fluidity than do the membranes from contact inhibited cells. Inbar and Shinitsky (Proc. Natl. Acad. Sci. U.S.A. 71: 2128, 1974) have used a fluorescent probe technique to demonstrate that membranes isolated from an ascites form of mouse malignant transformed lymphoma cells have a higher degree of fluidity than membranes isolated from normal lymphocytes. Our studies were undertaken to determine whether such differences in membrane fluidity could be demonstrated in chick embryo fibroblasts infected with a temperature sensitive mutant of Rous sarcoma virus. These cells, which appear normal when cultured at 41°, undergo transformation when the incubation temperature is shifted from 41° to 37°.

Cells were incubated with stearic acid label I (12,3) at a concentration of $1 \times 10^{-5}$ M for 30 min at 37°. The cells were then washed twice in buffer and then placed in a glass capillary tube. The electron spin resonance spectrum of the spin-labeled cells was recorded in a Varian E-4 spectrometer equipped with a variable temperature accessory. The fluidity of the cell membranes was expressed in terms of an order parameter S (Seelig, J. Am. Chem. Soc. 92: 3881, 1970), which is defined by the relationship

$$S = \frac{0.568\ (T_{\shortparallel}' - T_{\perp}')}{a'}$$



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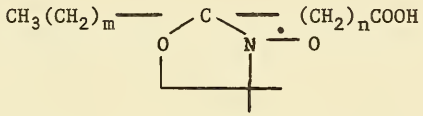
where

$$a' = \frac{1}{3}(T_{\parallel}' + 2T_{\perp}')$$

and $T_{\parallel}'$, $T_{\perp}'$ are the separations of the inner and outer hyperfine extrema of the membrane bound stearic acid label I (12,3).



I (m,n)

The membrane fluidity of chick embryo fibroblasts transformed by a number of viruses is shown in Table 1. It can readily be seen that there is no difference in the membrane fluidity, as measured by the order parameter, of the normal and transformed cells. We then attempted to reproduce the experiments of Barnett and co-workers using the same tumor cell lines. It can be seen from Table 1 that the membrane fluidity of mast cells from Balb/3T3 mice is unaffected by transformation with either murine sarcoma virus or SV40 virus. Since the completion of this work, other investigators have also reported their inability to demonstrate differences in membrane fluidity between normal and transformed cells using the spin label technique (Gaffney, Proc. Natl. Acad. Sci. U.S.A. 72: 664, 1975).

Transformed cells are known to exhibit certain differences in their physical and biochemical characteristics. Nevertheless, the suggestion by Barnett and co-workers and Inbar and Shinitsky that the membranes of transformed cells are more fluid than those from contact inhibited cells is not supported by these investigations. No further studies on the membrane properties of normal and transformed cells are contemplated at this time.

Keyword Descriptors: Spin labeling, electron spin resonance, membranes, membrane fluidity, chick embryo fibroblasts, Rous sarcoma virus, murine sarcoma virus, SV40 virus, mouse Balb/3T3 mast cells.

Honors and Awards: None

Publications: None

TABLE 1

Membrane fluidity of contact inhibited and transformed cell lines

| Cell line | Transforming agent | Temperature of growth (°C) | Temperature of ESR measurement (°C) | Order Parameter (S) |
|---|---|---|---|---|
| Chick embryo fibroblasts | -- | 37 | 37 | 0.60 |
| | | | 41 | 0.59 |
| " " " | Rous sarcoma virus BH (Bryan high titer strain) | 37 | 37 | 0.62 |
| | | | 41 | 0.60 |
| " " " | Rous sarcoma virus $SR_{50}$ (Schmidt-Ruppin strain) | 37 | 37 | 0.60 |
| | | | 41 | 0.59 |
| " " " | Rous sarcoma virus BHTA (temperature sensitive mutant of BH) | 37 | 37 | 0.60 |
| " " " | Rous sarcoma virus BHTA (temperature sensitive mutant of BH) | 41 | 41 | 0.61 |
| Mast cells from Balb/3T3 | -- | 37 | 37 | 0.61 (0.60)* |
| " " " " | Murine sarcoma virus | 37 | 37 | 0.61 (0.54)* |
| " " " " | SV40 virus | 37 | 37 | 0.61 (0.54)* |

* The values given in parentheses are the order parameters determined by Barnett and co-workers (Proc. Natl. Acad. Sci. U.S.A. 71: 1992, 1974).

Project No. Z01 HL 02509-01 PB
1. Pulmonary
2. Molecular Pharmacology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Acridine Spin Labels as Probes for Nucleic Acids

Previous Serial Number: None

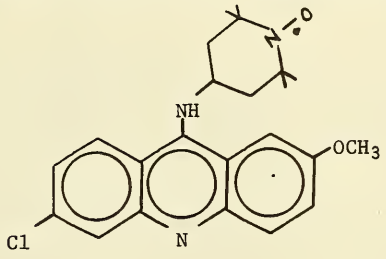
Principal Investigator: Dr. Birandra K. Sinha

Other Investigators: Dr. Colin F. Chignell
Dr. David B. Millar

Cooperating Units: Dr. Sinha held a Visiting Fellowship in the NHLI and is now a Guest Worker supported by the Microbiological Associates, Bethesda, Md.
Dr. Millar is Chief of the Laboratory of Physical Biochemistry, Environmental Biosciences Department, Naval Medical Research Institute, National Naval Medical Center, Bethesda, Md.

Project Description:

The nature of the interaction between amino acridines and nucleic acids is of considerable interest because of the mutagenicity of these compounds and their reported antitumor activity. The biological properties of the acridines are believed to arise either from their intercalation into DNA (or RNA) or from their association with phosphate groups on the outside of the double helix of nucleic acids. We have therefore synthesized amino acridine spin labels I and II and studied their interaction with RNA and DNA by means of electron spin resonance and other spectroscopic techniques.

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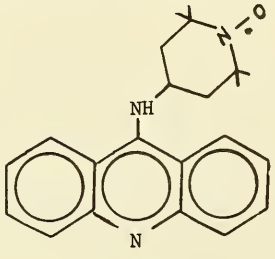


II



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Acridine spin labels I and II were synthesized from their corresponding 9-chloro-acridines and 4-amino-2,2,6,6-tetramethyl-1-piperidinyloxyl. Calf thymus DNA and calf liver RNA were purchased from a commercial source and used as received. Electron spin resonance (ESR) spectra were recorded with a Varian E-4 spectrometer operating at 9.5 GHz. Samples were introduced into the cavity in quartz micro flat cells. The temperature of the samples was maintained with a Varian variable temperature accessory and was measured with a Yellow Springs Instrument Co. telethermometer. A Cary 15 spectrophotometer equipped with a water jacket was used for the $T_m'$ determinations. Sedimentation velocity measurements were made with a Beckman Spinco Model E analytical ultracentrifuge.

The ESR spectrum of acridine spin label I bound to DNA was broad and asymmetric with a maximal hyperfine splitting ($2T_{\prime\prime}$) of 58.7 G. Neither heat denaturation of the DNA nor the addition of salt (0.1 M NaCl) altered the $2T_{\prime\prime}$ value of bound label I (Table 1). The $2T_{\prime\prime}$ value of acridine spin label II bound to DNA was 55.5 G. The addition of 0.1 NaCl to the DNA-II complex did not significantly alter the ESR spectrum of the probe (Table 1).

TABLE 1

The maximal hyperfine splittings ($2T_m$) of acridine spin labels I and II bound to calf thymus DNA

| Sample | NaCl (0.1 M) | $2T_{\prime\prime}$ (G) |
|---|---|---|
| DNA + I | - | 58.7 |
| DNA (heat denatured) + I | - | 59.0 |
| DNA + I | + | 59.0 |
| DNA + II | - | 55.5 |
| DNA + II | + | 55.0 |

In the absence of spin label, DNA was found to have a sedimentation coefficient of 11.2 S while on the addition of spin label I, the sedimentation coefficient decreased to 8.92. This observation suggests that label I is indeed intercalated into the DNA. The effect of the acridine spin labels on the melting temperature ($T_m'$) of DNA was studied by means of absorption spectroscopy and electron spin resonance. The $T_m'$ value for DNA alone was found to be 66.5° as determined by the increase in the optical density of the solution at 260 nm. In the presence of spin label I, the melting temperature of DNA was raised to 70°. It was also found possible to measure the $T_m'$ value for complexes between the spin labels I and II and DNA by monitoring the concentration of free spin label in solution as a function of temperature. The $T_m'$ value of a complex between DNA and I was found to be 69°

by this technique. The complex between DNA and II exhibited $T_m'$ values of 76° by the spectrophotometric assay and 78° by the ESR method.

The binding parameters for the interaction between I and II and DNA were determined by titrating a fixed amount of nucleic acid with increments of spin label and measuring the concentration of free probe by ESR. Both spin labels exhibited at least two kinds of binding sites on DNA (Table 2). At low ionic strength, it is known that binding occurs both by intercalation and an electrostatic interaction between the dye and the nucleic acid. The addition of a high concentration of salt is reported to abolish binding due to the electrostatic process. It will be seen from Table 2 that in the presence of 0.1 M NaCl the affinity of both spin labels for DNA is drastically reduced.

TABLE 2

| Sample | Binding Parameters | | | |
|---|---|---|---|---|
| | $N_1$ | $K_1$ ($M \times 10^{-5}$) | $N_2$ | $K_2$ ($M \times 10^{-5}$) |
| I | 0.150 | 120.0 | 2.11 | 0.74 |
| I + 0.1 M NaCl | 0.127 | 16.1 | 1.88 | 0.079 |
| II | 0.186 | 93.0 | 0.18 | 0.27 |
| II + 0.1 M NaCl | 0.163 | 19.7 | 0.22 | 0.22 |

From the ESR spectra of complexes between labels I and II and DNA, it is apparent that the piperidine ring that bears the nitroxide has greater mobility in the complex between DNA and II. This suggests that there is a fundamental difference in the way that these two spin labels interact with DNA. This difference is also reflected in the melting temperatures of complexes between I and II and DNA with spin label II producing a greater increase in the $T_m'$ value. Molecular models of labels I and II demonstrate a pronounced steric effect of the methoxyl group in the 2 position which forces the acridine ring into a conformation in which it is perpendicular to the flat piperidine ring. This observation may explain the differences in the mobility of the nitroxide group of labels I and II bound to DNA. It is also possible that the conformational differences between the acridine labels may explain why I is more effective in raising the melting temperature of DNA.

The ESR spectrum of label I bound to calf liver RNA indicated that the spin label had a higher degree of mobility than when bound to DNA. It is likely that the calf liver RNA exists in a single stranded form. It therefore appears possible that these spin labels may be useful in detecting double helical and single stranded regions in nucleic acids.

Acridines are of interest for two reasons: firstly, because of their biological and pharmacological effects and secondly, because they may be useful tools for probing the conformation of nucleic acids in biologically important complexes such as chromatin. In both these areas, it is to be expected that spin labels I and II will provide useful information not only on the pharmacological and toxicological effect of acridines in biological systems but also in the area of gene transcription. For future studies, 9-aminoacridines will be prepared in which different chain lengths are inserted between the 9-amino function and the acridine moiety. The binding of spin labels I and II to nucleic acids isolated from normal and transformed cells and to chromatin will also be studied.

Keyword Descriptors: Electron spin resonance, spin labels, 9-aminoacridines, DNA, RNA.

Honors and Awards: None

Publications: None

Project No. Z01 HL 02510-01 PB
1. Pulmonary
2. Molecular Pharmacology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Spin Label Studies of Halobacterium halobium Purple Membranes

Previous Serial Number: None

Principal Investigator: Dr. Colin F. Chignell

Other Investigator: Dr. Derek A. Chignell

Cooperating Unit: Dr. Derek A. Chignell is a Lecturer in Biochemistry in the Department of Biochemistry, The University, Dundee, Scotland

Project Description:

The isolation of a purple colored fragment from the cell membrane of the extreme halophile H. halobium represents one of the first successful attempts to obtain simple membrane system capable of translocating hydrogen ions (Oesterhelt and Stoeckenius, Proc. Natl. Acad. Sci. U.S.A. 70: 2853, 1973). These purple membranes (PM) contain a single protein, bacteriorhodopsin, which constitutes about 75% of their dry weight. The purple color ($\varepsilon_{max}$ 570 nm) of PM is due to retinaldehyde bound covalently to bacteriorhodopsin, and evidence has accumulated that this light-induced, proton-locating membrane bears a close relationship to the visual receptor membrane, although the ions translocated are different. Cone (Nature New Biol. 236: 39, 1972) and Brown (Nature New Biol. 236: 35, 1972) have demonstrated that the rhodopsin molecules in rod outer segments are highly mobile, the viscosity of the phospholipids being low enough to allow rotation and translation of the protein molecules within the membrane. This is consistent with a low degree of saturation of the fatty acid side chains found in the rod outer segment phospholipids. By contrast, the side chains of PM phospholipids are highly saturated, consisting of dihydrophytol groups linked to phosphatidylglycerol by ether, instead of ester, linkages. The degree of fluidity of PM therefore is of some interest.

Spin labels have been found to be extremely useful as probes of membrane structure (Jost and Griffith, Methods in Pharmacology, Vol. 2, 223, 1972). In particular, spin-labeled fatty acids with the general formula I (m,n) have provided information concerning the molecular organization, phase transitions and fluidity of various natural and synthetic membranes. The structure of mammalian visual receptor membranes has also been studied with the aid of



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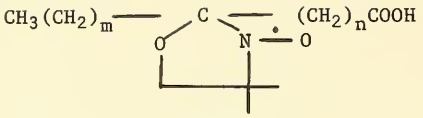
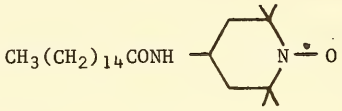


I (m,n)

spin labels (Hong and Hubbell, Proc. Natl. Acad. Sci. U.S.A. 69: 2617, 1972). We have therefore studied the molecular organization of PM with the aid of stearic acid spin labels (I 12,3; 5,10; 1,14) and a palmitamide spin label (II).



II

Stearic label I (12,3) exhibited maximum hyperfine splittings ($2T_m$) of 62 G and 59 G when bound to PM at 25° and 37°, respectively. A comparison of the $2T_m$ of I (12,3) bound to PM with the $2T_m$ of the same label bound to other membrane systems, such as human erythrocytes and lymphocytes, influenza virus, sarcoplasmic reticulum vesicles, E. coli membrane vesicles, and submitochondrial particles, indicates that the PM is the most rigid of all the membranes hitherto probed with this label. An Arrhenius plot of the $2T_m$ values of I (12,3) bound to PM showed a single discontinuity at about 29°. When the PM were crosslinked with glutaraldehyde, the Arrhenius plot became monophasic. Thus, it would appear that discontinuity in the Arrhenius plot of untreated PM is due to a phase change in bacteriorhodopsin rather than to an alteration in the molecular organization of the phospholipids.

Stearic acid spin label I (5,10) was also highly immobilized when bound to PM with $2T_m$ values of 62.7 G, 60.7 G and 58.7 G at 5°, 25° and 37°, respectively. In other membrane systems, the motion of stearic spin label I (5,10) has been found to be almost isotropic. In contrast, at 37° the $2T_m$ values of spin label I (12,3) and I (5,10) bound to PM are almost identical. Thus, it would appear that the rigidity of the PM system exists deep into the interior of the membrane. The electron spin resonance (ESR) spectrum of stearic acid label I (1,14) bound to PM at 25° revealed the presence of two populations of spin labels, one of which was more highly immobilized than the other. It seems likely that the highly immobilized labels are bound to boundary lipid which is closely associated with the bacteriorhodopsin (Biochim. Biophys. Acta 311: 141, 1973).

Verma and co-workers have been able to demonstrate a light-dependent change in the viscosity of beef retinal rods with the aid of a stearamide spin label analogous to II (Biochem. Biophys. Res. Commun. 55: 704, 1973). We were unable to detect any such changes in PM with labels I (1,14) or II. This is

perhaps not surprising, since it is known for PM the "dark reaction" in which the "bleached" form ($\epsilon_{max}$ 415 nm) returns to the "unbleached". form ($\epsilon_{max}$ 570 nm) is very fast (Nature New Biol. 233: 149, 1971). The dark reaction can be slowed by suspending the PM in a high salt solution saturated with ether. We found, however, that such treatment causes a "fluidization" of the membrane lipids as reflected by a dramatic increase in the mobility of PM bound label I (1,14). While illumination of such PM did result in bleaching, no change in the ESR spectrum of I (1,14) was detected. The dark reaction can also be slowed by cooling the PM in liquid nitrogen. At this temperature, the ESR spectrum of I (1,14) approached the "rigid glass" limit and was not affected by illumination.

The evidence presented in these studies points to an extremely rigid structure for PM with two populations of phospholipids, one of which is tightly bound to the protein in the membrane. Immobilization of the protein by glutaraldehyde has very little effect on this rigidity. Consistent with the findings of Racker and Hinkle (J. Membrane Biol. 17: 181, 1974), these experiments suggest that proton translocation occurs via a pore mechanism rather than being dependent upon the mobility of the bacteriorhodopsin within the PM. No change in the molecular organization of PM could be detected upon illumination.

This work has now been completed and no further studies on the purple membrane system from Halobacterium halobium are contemplated.

Keyword Descriptors: Purple membranes, Halobacterium halobium, spin labels, electron spin resonance.

Honors and Awards: None

Publications:

Chignell, C.F. and Chignell, D.A.: A spin label study of purple membranes from Halobacterium halobium. Biochem. Biophys. Res. Commun. 62: 136-143, 1975.

Project No. Z01 HL 02511-02 PB
1. Pulmonary
2. Molecular Pharmacology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Physicochemical Studies of Lung Surfactant Lipoprotein

Previous Serial Number: NHLI-217

Principal Investigator: Dr. Colin F. Chignell

Other Investigators: Mr. Robert Sik
Mr. Edward Sokoloski

Cooperating Unit: Mr. Sokoloski is in the Laboratory of Chemistry, NHLI

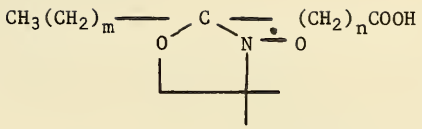
Project Description:

Lung surfactant is a lipoprotein which is known to reduce the surface tension of the alveolar lining of the lung, thereby preventing the collapse of this organ. Considerable controversy still persists concerning the precise function of the apoprotein portion of lung surfactant. This study has been undertaken to determine whether the apoprotein modifies the physical properties of lung surfactant.

Lung surfactant has been isolated from a lavage of the lungs of both dogs and rabbits. The lung surfactant lipoprotein was purified by means of density gradient centrifugation on NaBr gradients (Am. J. Physiol. 223: 707, 1972).

The surfactant lipoprotein, isolated from both rabbit and dog lungs, was delipidated by repeated treatment at $-20^{\circ}$ with ether ethanol (3:1). Disc gel electrophoresis of the apoprotein indicated the presence of two main components with masses of about approximately 30,000 daltons and 10,000 daltons. Both preparations were found to be contaminated with plasma proteins, of which serum albumin seemed to be the main component.

Electron spin resonance studies with the aid of stearic acid spin labels I (m,n) have indicated that the molecular organization of the intact lipoprotein is highly ordered at or near the interface with the aqueous environment but that the interior of the lipoprotein is extremely fluid. In these respects, lung surfactant lipoprotein resembles the erythrocyte ghost membrane in terms of its molecular organization.



I (m,n)

Nuclear magnetic resonance (NMR) measurements of the $P^{31}$ resonance of the phospholipid phosphate groups present in the lipoprotein have been attempted. However, it was found that the $P^{31}$ resonance of lung surfactant was broadened to the point where it was undetectable in the NMR spectrometer. In contrast, when the phospholipids were extracted from the lipoprotein and resonicated into deuterium oxide, a sharp $P^{31}$ resonance was observed. These results suggest that in the lung surfactant lipoprotein the phosphate groups of the phospholipids are highly immobilized.

Lung surfactant plays an important role in lung physiology. Furthermore, alterations in lung surfactant in certain disease states and after exposure to environmental pollutants are known to cause respiratory problems. It is therefore important to understand more precisely the physical characteristics of this important lipoprotein.

The physical characteristics of the lung surfactant lipoprotein will be further characterized by means of other spin labels. In addition, the physical properties of the lung surfactant lipoprotein will be compared with those of isolated sonicated phospholipids in an attempt to determine what effect, if any, the apoproteins have on the physical characteristics of this complex.

Keyword Descriptors: Lung surfactant, apoprotein, phospholipids, electron spin resonance, spin labels, nuclear magnetic resonance, gel electrophoresis.

Honors and Awards: None

Publications: None

Project No. Z01 HL 02512-02 PB
1. Pulmonary
2. Molecular Pharmacology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Further Spin Label Studies of Egg White Avidin

Previous Serial Number: NHLI-100

Principal Investigator: Dr. Colin F. Chignell

Other Investigator: Dr. Birandra K. Sinha

Cooperating Unit: Dr. Sinha held a Visiting Fellowship, NHLI, and is now a Guest Worker supported by the Microbiological Associates, Bethesda, Md.

Project Description:

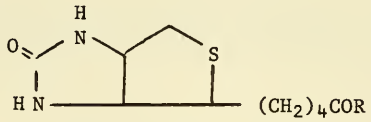
Avidin is a tetrameric protein (mass 68,000 daltons) that binds four molecules of vitamin biotin (I). The biotin binding sites, one per subunit, are grouped in two pairs at opposite ends of the avidin molecule (Green et al., Biochem. J. 125: 781, 1971). We have studied the topography of the avidin binding sites with the aid of four spin-labeled analogs of biotin (II-V). Fluorescence and optical absorption spectroscopy indicated that labels II-V occupied the same binding sites on avidin as did biotin. The electron spin resonance spectrum (ESR) of the 4:1 complex between II and avidin contained broad line components characteristic of a highly immobilized spin label (Table 1). Dipole-dipole interactions between spin labels bound to adjacent sites split each of the major hyperfine lines into doublets with a separation of 13.8 G. The distance between adjacent bound nitroxide groups was calculated from the splitting to be 16 A (Table 1). The dissociation of the 4:1 complex between II and avidin was biphasic with approximately half of the spin labels dissociating at a rate ($k_{diss} = 2.51 \times 10^{-4}$ $sec^{-1}$) that was much faster than the remainder ($k_{diss} = 1.22 \times 10^{-5}$ $sec^{-1}$). The electron spin resonance spectrum of the 2:1 complex between II and avidin clearly showed that immediately after mixing spin labels were distributed in a random fashion among the available binding sites but that they slowly redistributed themselves so that each label bound to a site which was adjacent to an occupied site. The final time-independent electron spin resonance spectrum exhibited a splitting of 69 G between the low and high field hyperfine lines which is characteristic of highly immobilized, noninteracting spin label. Spin labels III and IV interacted with avidin in a similar fashion to that described for II with the exception that their dipolar splittings were 11.9 G and 14.2 G, respectively. From these splittings it was estimated that the distance between adjacent avidin bound nitroxides was 16.7 A for label III and 15.7 A

TABLE 1

The ESR parameters of complexes between the spin-labeled biotin analogs and avidin

| Compound | Maximum hyperfine splitting of 2:1 complex* (G) | Dipolar splitting of 4:1 complex (G) | Calculated distance between adjacent nitroxides (Å) |
|---|---|---|---|
| II | 69.0 | 13.8 | 16.0 |
| III | 64.9 | 11.9 | 16.7 |
| IV | 63.5 | 14.2 | 15.7 |
| V | 62.0 | -- | -- |

* Measured at least 3 hr after mixing.



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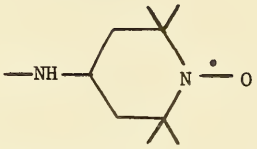
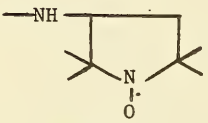
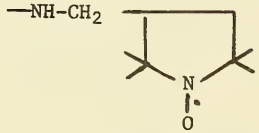
II R =



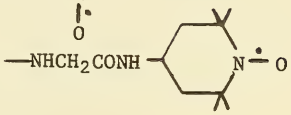
III R =



IV R =



V R =





775

for label IV (Table 1). The electron spin resonance spectrum of label V bound to avidin was characteristic of a noninteracting, highly immobilized nitroxide with a maximum splitting of 62 G. The electron spin resonance spectrum of V bound to avidin was independent of both time and the amount of bound label. The rate of dissociation of V from a 4:1 complex with avidin was monophasic ($k_{diss} = 3.85 \times 10^{-5}$ $sec^{-1}$). These studies support a model for avidin in which the recognition site of the heterocyclic ring system of biotin is represented as a cleft located within a hydrophobic depression in the surface of the protein.

These studies clearly show that spin-labeled ligands can provide useful information on the topography of receptors and other binding sites found in proteins and other biologically important macromolecules. The biotin avidin system is the first in which it has been possible to estimate molecular distances using the electron spin resonance technique and spin labels. It is hoped that a more precise knowledge of binding site topography may make it possible to design more specific drug molecules.

This project has now been completed and no further work on this system is anticipated.

Keyword Descriptors: Avidin, biotin, electron spin resonance, fluorescence spectroscopy, absorption spectroscopy, spin labels.

Honors and Awards: None

Publications: Chignell, C.F., Starkweather, D.K. and Sinha, B.K.: A spin label study of egg white avidin. J. Biol. Chem., in press.

Project No. Z01 HL 02513-03 PB
1. Pulmonary
2. Molecular Pharmacology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Purification and Characterization of $Na^+ + K^+$-ATPase

Previous Serial Number: NHLI-97

Principal Investigators: Dr. Clyde A. Takeguchi
Dr. Ueli Honegger
Dr. Elwood O. Titus

Other Investigator: Mr. Wallace W. Holland

Cooperating Units: Dr. Takeguchi is a Research Associate in the Pharmacology-Toxicology Program, NIGMS
Dr. Honegger holds a Visiting Fellowship, NHLI

Project Description:

Objectives: The $Mg^{++}$-dependent, $Na^+ + K^+$-stimulated, ouabain-inhibitable ATP hydrolyzing system is an integral part of the active transport mechanism that moves cations across biological membranes against electrical and chemical gradients. The enzyme is a lipoprotein complex which is itself a part of the cell membrane. The goals of this project are to describe the molecular events (presumably reversible changes in conformation of ATPase) that are associated with ion transport and to ascertain those structural features which enable the ionophoric portions of the system to distinguish between sodium and other monovalent cations. Previously this laboratory has demonstrated that both agents that act extracellularly (cardiac glycosides and potassium) and those which act intracellularly (sodium and ATP) alter the conformation of a single protein which appears to traverse the whole membrane. Certain of these conformational changes apparently expose normally inaccessible protein sulfhydryl groups. The present study was therefore designed to seek reagents that could specifically label the conformationally mobile sulfur-containing moieties or that might initiate the cleavage of peptide chains at these sites. Since, in theory, both of these objectives could be obtained by cyanylation of the SH groups of conformationally mobile cysteine residues (Degani and Patchornick, Biochemistry 13: 1, 1974), the reaction of the ATPase system with cyanylating reagents is being examined. Methods for obtaining highly purified enzyme in bulk are also being examined.

Methods Employed: The outer medulla of the rabbit kidney, which is particularly rich in transport sites associated with sodium reabsorption,

has been used as the enzyme source. A modification of the Jorgensen procedure (Methods in Enzymology 32: 277, 1974) was employed to obtain purified ATPase. TCNB (5-thiocyano-2-nitrobenzoic acid) was synthesized according to the method of Degani and associates (J. Am. Chem. Soc. 92: 6969, 1970).

Major Findings: A modification of the method of Jorgensen consistently resulted in high yields of ATPase with high specific activity from rabbit kidneys obtained from commercial sources.

$5 \times 10^{-4}$ M Ellman's reagent (5,5-bisdithio-2-nitrobenzoic acid) and $5 \times 10^{-3}$ M TCNB inhibited ATPase specific activity by 54% and 68%, respectively. Kinetic experiments with these sulfhydryl reagents also indicated that there may be two populations of sulfhydryl groups in the active enzyme, one with a reaction t-1/2 of a few seconds, and the second with a t-1/2 of about 20 to 30 minutes. Na and ATP seem to protect the enzyme from inactivation by these sulfhydryl reagents. This protection is lost with the addition of $Mg^{++}$.

Significance to Biomedical Research and the Program of the Institute: The ionic gradients across cell membranes are maintained by this cation transport system. These gradients are a prerequisite for all cellular functions involving neuronal conduction, the production of electrical currents, the excitability of muscle and nerve, the storage of neurotransmitters that convey impulses from nerve to smooth muscle and the bulk transport of electrolytes. Attempts to characterize fully the enzymatic basis for this transport system are fundamental to an understanding of the regulatory mechanisms of the cardiovascular system. Purification and characterization of this enzyme system would permit further investigations into the role of various drugs, such as ouabain and other cardiac glycosides, upon this enzyme at the molecular level.

Proposed Course of Project: Further studies will focus on labeling the purified ATPase with $^{14}C$- and $^{13}C$-labeled TCNB. Since cyanylation initiates chain breakage, patterns of peptide fragments should reflect labeling differences due to ligand-dependent conformational changes. $^{13}C$ NMR spectroscopy may enable us to see the micro-environment of the labeled sulfhydryl groups as it undergoes conformational changes. Protein degradative studies will be undertaken in an effort to determine the location of the two populations of SH groups revealed by the kinetic studies.

Keyword Descriptors: ATPase, ion transport, membranes, cardiac glycosides, sulfhydryl groups

Honors and Awards: None

Publications: Titus, E.O. and Hart, W.M.,Jr.: The use of sulfhydryl reagents to identify proteins undergoing ligand-dependent conformational changes associated with the function of $(Na^+ + K^+)$-ATPase. Ann. N.Y. Acad. Sci. 242: 246-254, 1974.

Project No. Z01 HL 02514-01 PB
1. Pulmonary
2. Molecular Pharmacology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Chemical Characterization of Pharmacological Receptors

Previous Serial Number: None

Principal Investigator: Dr. Elwood O. Titus

Other Investigator: Mr. Wallace W. Holland

Cooperating Units: None

Project Description:

Objectives: The molecular mechanisms by which neurohumoral transmitters activate receptors in mammalian smooth muscle, secretory glands, etc., are unknown. The first step is almost certainly adsorption to a recognition site which exhibits high affinity for a limited range of organic structures related to the natural agonist. Conformational changes associated with this adsorption initiate the response. The goal of this project is to isolate those components of adrenergic and other receptors which serve as agonist-recognition sites in smooth muscle and other autonomically innervated mammalian tissues.

Studies of the nicotinic cholinergic receptor isolated from the electroplax of electric fish together with studies of insulin, angiotensin and other receptors from mammalian tissues indicate that individual receptors are characterized by specific proteins. The introduction of site-directed, covalently bound radioactive labels into such proteins should be useful in isolating receptors. Although the specific agonist-binding properties of the electroplax receptor survive solubilization in detergents and separation from the membrane matrix, studies with the mammalian muscarinic receptor indicate that this may not always be the case. Even if it is impossible to purify a receptor without denaturation, the isolation of a labeled polypeptide, unequivocally identifiable as derived from the receptor, is of interest, since the information which defines the receptor is inherent in the amino acid sequence of the protein.

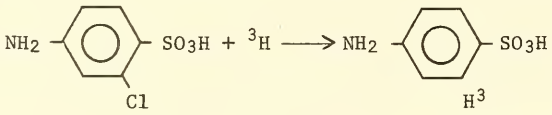
Methods Employed: The fractionation of membrane proteins will be followed by radioactive labels covalently attached to SH, COOH, or tyrosyl moieties since, with appropriate choice of reagents, these functional groups can be labeled in intact tissue under physiological conditions. Since the

adsorption of an agonist causes conformational perturbation of the receptor substance and since conformational change is frequently manifested in altered reactivity of the above-mentioned moieties, ligand-dependent changes in labeling patterns will be used for the identification of receptor-related proteins. The procedure is best controlled by a double isotope procedure in which identical preparations are treated with the same reagent, that reagent being labeled with different isotopes as it is used in the presence or the absence of the agonist which alters the conformation. The two preparations are mixed and the individual proteins are isolated from membranous subfractions. In theory, a constant ratio of the two isotopes should be observed for all proteins except those affected by the agonist. The procedure has been successfully used for the identification of the M protein of bacterial membranes (Fox and Kennedy, Proc. Natl. Acad. Sci. U.S.A. 54: 891, 1965), the ouabain-sensitive component of ion transport systems (Hart and Titus, J. Biol. Chem. 248: 4674, 1973), the glucagon-sensitive components of the adenylate cyclase system in rat liver (Storm and Chase, J. Biol. Chem. 250: 2539, 1975) and one of the proteins in the cholinergic receptor of electroplax (Reiter et al., Proc. Natl. Acad. Sci. U.S.A. 69: 1168, 1972).

Major Findings: A double isotope procedure applicable to the minute quantities of receptors in mammalian tissues will require radioactive labels with specific activities of the order of Curies per mmole. Both experience in this laboratory and a survey of the literature indicated that changes in the rate of reaction of protein sulfhydryl groups with electrophilic reagents were likely to be the most sensitive indicators of conformational alteration. First priority was therefore given to synthesis of reagents that reacted with SH groups, that could be easily labeled with two isotopes and that would react preferentially with extracellular sites.

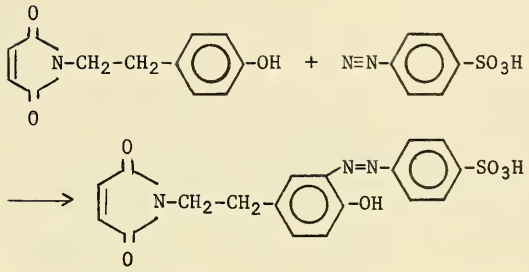
In collaboration with the New England Nuclear Corp., $^{3}H$-sulfanilic acid was prepared by reaction (1) and purified to a specific activity of 3 Ci/mmole

(1)




Since $S^{35}$-labeled sulfanilic acid of equivalent specific activity is available, and since coupling of diazotized anilic acid to protein SH, tyrosyl and imidazole groups occurs readily at physiological pH, sulfanilic acid will be used in double labeling experiments. Preliminary experiments on the relative effects of d- and l-norepinephrine on the labeling of intact rabbit aortas by diazotized $S^{35}$-sulfanilic acid indicate that individual proteins isolated from the membrane fraction accept from 0.03 to 0.9 pmols of label and that two proteins from this fraction may be sensitive to norepinephrine.

The label from sulfanilic acid may be introduced into a reagent more specific for SH groups by the following reaction:



A synthesis of the starting material, N-hydroxyphenylethylmaleimide, has been devised and is nearly completed.

Significance to Biomedical Research and the Program of the Institute: Clinically important differences in response to drugs of essentially similar structure probably reflect the existence of different receptor subtypes in different tissues. Specific examples are the $\beta_2$-adrenergic agonists such as salbutamol which selectively act on the bronchodilatory mechanisms in lung and the $H_2$-histamine antagonists which act selectively on histamine-dependent gastric acidification. The molecular basis for these distinctions and a more rational basis for the design of organ selective drugs would be provided by information on the chemical nature of receptors.

Proposed Course of Project: Double isotope studies of the labeling of adrenergic receptors in rabbit aorta and other tissues will be carried out using $S^{35}$- and $H^3$-labeled reagents. Reaction schemes specific for conformationally sensitive COOH groups will be designed.

Keyword Descriptors: Receptors, norepinephrine, radioactive labeling.

Honors and Awards: None

Publications: Titus, E.O.: Characterization of pharmacological receptors. Naunyn-Schmiedeberg's Archiv. Pharmakol., in press.

Project No. Z01 HL 02515-02 PB
1. Pulmonary
2. Molecular Pharmacology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Biochemical Effects of Paraquat on the Lung

Previous Serial Number: NHLI-105

Principal Investigators: Dr. Elise Ann Brandenburger Brown
Dr. Harriet M. Maling

Other Investigator: Mr. Wilfred Saul

Cooperating Unit: Dr. Maling and Mr. Saul are with the Laboratory of Chemical Pharmacology, NHLI

Project Description:

This project is a study of some biochemical effects which might be involved in the pulmonary toxicity of the herbicide, paraquat (Pq). This year we continued our studies on the effects of Pq and related bipyridinium herbicides on acetylcholinesterase (ACE) of the lung. With acetylthiocholine bromide as a substrate, as described by Ellman et al. (Biochem. Pharmacol. 7: 89, 1961), the apparent $K_i$ for Pq in rat lung homogenates was $2.9 \times 10^{-3}$ M. The $K_i$ values for diquat and morfamquat were $2.4 \times 10^{-4}$ M and $1.4 \times 10^{-4}$ M. Since the concentration of Pq found in rat lung under toxic conditions is more than an order of magnitude less than the apparent $K_i$ value, it is improbable that inhibition of ACE is responsible for the pulmonary edema induced by this compound.

Since some ACE inhibitors also block the neuromuscular junction and since Pq produces neuromuscular weakness in rats, we observed the effects of Pq in chicks. The i.p. injection of Pq (15 mg/kg) produced extensor paralysis and death within 6 hr; no obvious changes occurred after 10 mg/kg. A subthreshold dose of d-tubocurarine chloride (1.5 mg/kg) produced a transient flaccid paralysis when administered 90 min after 10 mg/kg of Pq. Agents that depolarize the neuromuscular junction cause an extensor paralysis, and competitive blocking agents produce a flaccid paralysis in birds (Paton and Zaimis, Pharmacol. Rev. 4: 219, 1952). Our observations suggest that Pq can produce a dual block of neuromuscular function.

Many compounds have been tested for protection against the pulmonary toxicity of Pq (H.M. Maling, unpublished). Among the few effective compounds were beta adrenergic receptor blocking agents, especially dl-propranolol (Pr). In order to explain why Pr increases the $LD_{50}$ for Pq in rats, we examined the

effects of Pr, Pq, and Pr + Pq on the incorporation of $^{32}$P-phosphate into the phospholipids of rat lung slices. A study of phospholipid synthesis seemed desirable, since Pq has been reported to lower the quantity of dipalmitoyl phosphatidylcholine, the predominant pulmonary surfactant (Fisher et al., J. Appl. Physiol. 35: 268, 1973). Furthermore, the pulmonary edema produced by Pq could result from alterations in membrane permeability, which is maintained in part by phospholipids. Rat lung slices were preincubated with Pr ($5 \times 10^{-4}$ M), Pq ($5 \times 10^{-4}$ M), or Pr + Pq for 30 min before the addition of $^{32}$P-phosphate and continued incubation for an additional 30 min. The pattern of incorporation into phospholipids was altered by both Pr and Pq but more markedly by Pr. The incorporation into phosphatidylglycerol was doubled by Pr, reduced slightly by Pq, and increased about 50% by Pr + Pq. Incorporation into phosphatidylcholine was reduced slightly by Pq, about 70% by Pr, and about 80% by Pr + Pq. In contrast, incorporation into phosphatidic acid was increased about 50% by Pq, fivefold by Pr, and sixfold by Pr + Pq. Incorporation into another phospholipid, presumably cytidine diphosphate-diglyceride (Hauser and Eichberg, J. Biol. Chem. 250: 105, 1975), was increased more than twelvefold by Pr or Pr + Pq but was unaffected by Pq alone. These changes in metabolism may be related to the protective effects of treatment with Pr.

A variety of radiolabeled precursors, such as cytidine, myoinositol, and sodium palmitate, will be utilized in further studies of the alterations in pulmonary phospholipids produced by Pr and Pq. Anenoic phospholipids will be determined because of their probable relationship to pulmonary surfactant.

Keyword Descriptors: Paraquat, diquat, morfamquat, propranolol, lung phospholipids, acetylcholinesterase of lung, phosphatidic acid, phosphatidylglycerol, cytidine diphosphate-diglyceride, pulmonary surfactant, phosphatidylcholine

Honors and Awards: None

Publications:

Maling, H.M., Eichelbaum, F.M., Saul, W., Sipes, I.G., Brown, E.A.B. and Gillette, J.R.: The nature of the protection against $CCl_4$ toxicity produced by pretreatment with Dibenamine (N-(2-chloro-ethyl) dibenzylamine). Biochem. Pharmacol. 23: 1479-1491, 1974.

Trams, E.G. and Brown, E.A.B.: The activity of 2', 3'-cyclic adenosine monophosphate 3'-phosphoesterhydrolase in elasmobranch and teleost brain. Comp. Biochem. Physiol. 48B: 185-189, 1974.

Project No. Z01 HL 02516-01 PB
1. Pulmonary
2. Molecular Pharmacology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Information Retrieval in Pharmacology

Previous Serial Number: None

Principal Investigator: Dr. Elise Ann Brown

Other Investigator: Dr. Harry Keiser

Cooperating Unit: Dr. Keiser is Deputy Chief of the Hypertension-Endocrine Branch and Head, Section on Experimental Therapeutics, HE, NHLI

Project Description:

In initiating new research projects, information must be obtained on prior efforts in the field, on the feasibility of available procedures and on the extent to which the phenomena of interest have been satisfactorily explored. Such assessments of the state of the art require a professional background not supplied by computer personnel or technical librarians. This is especially true in clinical and pharmacological studies, where judgments must frequently be made concerning dose, species, routes of administration of drugs, etc. Information retrieval and appraisal activities have therefore been established in NHLI.

Extensive use was made of the MEDLINE and TOXLINE computer files on line from the National Library of Medicine, the personal files of various investigators and interviews with informed investigators. I attended a course on the use of TOXLINE in order to improve my facility with the computer systems.

Major inquiries were requested by ten investigators and minor inquiries by four investigators. All but one were personnel of the National Heart and Lung Institute.

The areas of biomedical investigation which were explored included

1) Propranolol, i.v. administration and half-life in man
2) Paraquat--mechanism of toxicity
3) Diquat toxicity
4) Slow reacting substance of the lung (SRS-A)--history, identity, source
5) Electron spin resonance studies



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6) Intercalation of DNA and RNA with acridine compounds
7) Adenosine pharmacodynamics in tissue cell systems
8) Phospholipid formation in the lung
   a) formation of dipalmitoyl lecithin
   b) formation of cytidine diphosphatediglyceride
   c) formation of phosphatidylethanolamine
   d) effects of 5-hydroxytryptamine, dopamine and acetylcholine
   e) changes with changes in cell membrane permeability
9) Sulfur compounds--metabolism in the lung
10) Drug-metabolizing enzymes in the lung
11) Effect of isoproterenol on DNA synthesis.
12) Glutathione levels in the lung as altered by drugs
13) Mucopolysaccharides and protein binding sites in lung
14) Pharmacology and adverse effects of
   a) Phenformin
   b) Ibuprofen
   c) Primaquine
   d) Ozone
   e) Minoxidil
   f) Pindalol
15) Spectral analysis of retinal pigments
16) Use of superoxide dismutase in therapy
17) Protein synthesis in the lung
18) Antihypertensive effects of SQ 20881
19) Biological effects of Pepstatin
20) Interaction of hexamethonium and acetylcholinesterase
21) Verbenol as an hypertensive agent
22) Effects of conconavalin A and plant agglutinins on cell membrane permeability and on ATPase
23) Enzymes of histamine metabolism in monkey lung
24) Lipoperoxidation in lung

The volume of medical literature has increased so that individuals cannot handsearch for topics easily. Rapid access to information on the usage and/or toxicology of compounds is essential for the most cost effective utilization of biomedical personnel.

Collaboration has been actively sought with several NHLI laboratories. I have instructed several investigators in search procedures so that they can expedite their own work.

In order to double the output rate of computer searches, a proposal for a new cathode ray terminal system was submitted in January to Dr. H. Keiser.

The proposed system will double the rate for entering commands to the MEDLINE and TOXLINE systems and increase by 8-fold the rate of interaction with DCRT computers. This should encourage more use of the facility and reduce the time needed to obtain information.

Keyword Descriptors: Information retrieval, drug metabolism, pharmacology

Honors and Awards: None

Publications:

Brown, E.A.B.: The localization, metabolism, and effects of drugs and toxicants in lung. Drug Metab. Rev. 3: 33-87, 1974.

ANNUAL REPORT OF THE
CLINIC OF SURGERY
NATIONAL HEART AND LUNG INSTITUTE
July 1, 1974 through June 30, 1975

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.

Operative Treatment of Acquired Valvular Heart Disease. The first successful prosthetic replacement of the mitral valve was carried out in this Clinic in 1960. Since that time the study and treatment of patients with acquired valvular heart disease has continued as one of the principal clinical research efforts of the Surgery Branch. The majority of patients who have had mitral valve replacement have had insertion of a prosthesis with a moving poppet of ball or disc configuration. The hemodynamic function of such artificial valves is generally satisfactory, but their use has been associated with an extremely high incidence of systemic emboli. The incidence of emboli can be reduced but not abolished by the administration of anticoagulants or agents which decrease platelet adhesiveness. Laboratory investigations, here and at other centers, indicated that a valve constructed of autologous or homologous tissue would be superior to a mechanical device. Experimental and clinical use of tissue valves, however, soon proved that the attachment of the tissue to the supporting framework was insecure and valve failure from detachment universally occurred. Studies in this laboratory proved that if the supporting frame for the tissue was made flexible rather than rigid that the stresses on the attachments were greatly reduced.

Thus, in the summer of 1965, the first porcine xenograft aortic valve was inserted in the mitral position. That patient is living and well. Since that time 110 additional patients have undergone mitral and/or tricuspid valve replacement with the porcine xenograft. Eighty-six patients are alive, cumulative followup approximates 2000 patient-months, and 15 patients have been followed more than 4 years. Anticoagulants have not been administered postoperatively unless their use was required by a rigid prosthesis in the aortic position. One patient only has had a systemic embolus and this occurred in the immediate postoperative period presumably from the Teflon valve base. The function of the xenograft has been studied by postoperative cardiac catheterization in 44 patients. Following mitral replacement the average left atrial mean pressure was 17 mm. Hg, the average atrio-ventricular diastolic gradient 4 mm. Hg, and the average mitral valve area was 2.3 $cm.^2$. After tricuspid replacement the average right atrial mean pressure was 11 mm. Hg, and the right atrio-ventricular pressure gradient averaged 4 mm. Hg. Valve failure, with severe regurgitation through the xenograft has occurred in one patient, 57 months following operation. The defective valve was removed and dysfunction proved to be

the result of infection with a fungus, probably histoplasmosis. Two patients have had bacteremia and have been successfully treated with antibiotics; valve dysfunction did not result.

This clinical experience indicates that at this time the porcine xenograft, preserved in stabilized glutaraldehyde and mounted on a flexible stent, is the prosthesis of choice when replacement of the mitral or tricuspid valve is necessary. Such xenografts will not be utilized in the aortic position in this Clinic until evidence is available that the valves can withstand the additional mechanical stress which accompanies implantation in the aortic root.

Operative Management of Acquired Tricuspid Valve Disease. Tricuspid regurgitation commonly accompanies acquired mitral stenosis or regurgitation, and optimal management of the tricuspid disease has never been defined precisely. In 1967 it was the opinion of the surgeons in this Clinic that in virtually all patients tricuspid regurgitation would regress spontaneously if the mitral disease was dealt with effectively. Since that time clinical observations have indicated that that premise was often incorrect and a number of patients in whom tricuspid regurgitation was not treated have continued to experience severe symptoms of tricuspid regurgitation after mitral valve replacement. For this reason, the operative management of tricuspid disease has become more aggressive, and an increasing number of patients have undergone either tricuspid valve replacement or tricuspid annuloplasty.

During the years 1972-1974, 76 patients with mitral and/or aortic valve disease were proved to have tricuspid regurgitation or stenosis when the tricuspid valve was palpated at the time of operation. In 21 of the 76 patients no operative procedure on the tricuspid valve was carried out; in 30 patients a tricuspid annuloplasty was performed; in the remaining 25 patients the tricuspid valve was replaced with a procine xenograft. The operative mortality among patients in whom the tricuspid valve was not treated was 10%; in those having an annuloplasty mortality was 23%; and the operative mortality was 12% in the patients among the patients having tricuspid valve replacement. Clinical followup and hemodynamic evaluations indicate that tricuspid valve replacement gives a long-term result superior to that provided by tricuspid annuloplasty. Evaluation of those patients in whom tricuspid regurgitation was present but was not treated is difficult, since it is likely that the magnitude of regurgitation was less in this clinical subgroup than in those who had annuloplasty or valve replacement. It is clear, however, that an aggressive surgical approach is indicated when the patient with mitral valve disease is found to have significant tricuspid regurgitation as well, and is likely that the operation of choice is tricuspid valve replacement.

Intimal Proliferation in Venous Autografts. This year it seems likely that more than 200,000 patients will undergo operative treatment for obstructive coronary atherosclerosis. The operative procedure usually performed is the insertion of multiple segments of autologous saphaneous vein as grafts between the ascending aorta and the coronary arteries distal to the sites of principal obstruction. The benefit provided by such an

operation is largely dependent upon patency of the vein grafts. The application of precise microsurgical techniques will provide assurance of early patency, but in a significant number of patients the grafts become occluded after 6-18 months by progressive proliferation of the intima of the graft.

An experimental study was made of the effects of two pharmacologic agents on intimal proliferation in venous autografts. In dogs, 36 segments of the femoral artery were replaced with segments of autologous femoral vein. The animals were then divided into three groups: no specific post-operative treatment; postoperative administration of dipyridamole (Persantine); postoperative treatment with methylprednisolone. Six weeks after operation femoral arteriography was carried out to determine graft patency, and the animals were then killed and the grafts removed and studied histologically. Overall, 94% of the grafts were patent and the patency rate did not differ among the three groups. The thickness of the intima was measured in the proximal, middle, and distal thirds of the grafts to quantify the extent of intimal proliferation. Intimal proliferation was similar in the control and dipyridamole treated groups. Dogs which had been treated with methylprednisolone had significantly less intimal thickening in the middle third of the grafts than did control animals (125 microns vs. 214).

The experiment shows that at six weeks the extent of intimal proliferation is not influenced by dipyridamole treatment. While the steroid effectively decreased thickening in the middle of the grafts, substantial proliferation was still evident near each anastomosis. This suggests that unknown factors relating to blood flow and the technique of anastomosis may also be of importance. Furthermore, observations of this nature carried out over a period of one year would more closely approximate the clinical problem. The feasibility of such long-term studies is presently under investigation.

Experimental and Clinical Studies of the Contribution of Atrial Contraction to Right Heart Function Before and After Right Ventriculotomy. Many studies have been made of the effects of left atrial contraction on left ventricular function. Little attention, however, has been given to the contribution of right atrial systole to right ventricular function. The studies outlined were stimulated by the observation that many patients evidenced right heart failure when they developed ineffective atrial contraction after operations requiring right ventriculotomy (repair of tetralogy of Fallot, closure of ventricular septal defect).

The effects of atrial contraction were studied in open chest dogs before and after a vertical right ventriculotomy was made. Effective atrial contraction was abolished by simultaneous atrial and ventricular (A-V) pacing. Before ventriculotomy, and when cardiac output, aortic pressure, and heart rate were kept constant, the mean right atrial pressure rose only slightly (1.4 mm. Hg) when A-V pacing was instituted. In the same animals, however, right atrial pressure rose 9.5 mm. Hg after a right ventriculotomy had been made by a technique which did not require bypass or inflow occlusion. Right ventricular failure with gross evidences of tricuspid regurgitation was easily induced after ventriculotomy by volume overload and A-V pacing.

With the blood volume unchanged restoration of effective atrial contraction by sequential A-V pacing eliminated palpable tricuspid regurgitation and lowered the average mean right atrial pressure from 22 to 4 mm. Hg. After right ventriculotomy the right atrial pressure was maintained constant by adjustments of circulating blood volume. In these animals loss of atrial contraction was followed by a 42% reduction in cardiac output.

Additional observations were made in 13 patients who were studied in the immediate postoperative period. Both atrial and ventricular pacing electrodes had been placed at operation, permitting simultaneous or sequential pacing of the right atrium and right ventricle. In 8 patients who had had right ventriculotomies abolition of atrial contraction caused an average reduction in cardiac output of 22% (thermal dilution method). In five other patients in whom the right ventricle had not been incised simultaneous A-V pacing caused a trivial fall in cardiac output of only 5%.

The therapeutic implications of the study are clear. The right ventricular failure which is commonly observed after repair of the tetralogy can be ameliorated by insuring that effective atrial contraction is maintained. This requires that both atrial and ventricular electrodes be placed at operation so that during periods of nodal rhythm or complete heart block appropriately timed atrial contractions can be supplied artifically.

Project No. Z01 HL 02601-01 SU
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Cardiac valve replacement with the Hancock porcine: A five year clinical experience

Previous Serial Number: None

Principal Investigators: Charles L. McIntosh, M.D.
Lawrence L. Michaelis, M.D.
Andrew G. Morrow, M. D.

Project Description: The first clinical implantation of a glutaraldehyde-fixed porcine xenograft mounted on a flexible stent was performed in July 1970 at the NHLI. Since then, 110 patients have undergone single or multiple valve replacement with this prosthesis; 86 patients are alive. Cumulative followup totals 1732 patient months, with 10 patients followed more than four years. Anticoagulants have not been administered routinely postoperatively.

Results: Pre- and postoperative hemodynamic assessments have been performed in 44 patients. Following mitral valve replacement the average left atrial mean pressure was 17 mm. Hg; the average left atrio-ventricular mean diastolic gradient was 4 mm. Hg; the average calculated mitral valve area was 2.3 $cm^2$. After tricuspid valve replacement the average right atrial mean pressure was 11 mm. Hg, and the right atrio-ventricular mean pressure gradient was 4 mm. Hg. Prosthetic regurgitation secondary to prosthetic dysfunction has occurred in one patient 57 months following mitral valve replacement with a xenograft. Preliminary pathological examination revealed the valve to be infected with a fungus organism, most likely histoplasmosis. One patient has had an arterial embolus (10 days postoperative, good recovery). Two patients with documented bacteremia were successfully treated with antibiotics, and did not require replacement of their xenograft prosthesis.

Keywords: Porcine Xenograft, Long-term followup, heterograft

Proposed Course: This study will be presented at the Vascular Society Meeting in June 1975.

Project No. Z01 HL 02602-01 SU
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Cardiac patient data profile

Previous Serial Number: None

Principal Investigators: Charles L. McIntosh, M.D.
Andrew G. Morrow, M. D.

Cooperating Unit: Data Management Branch, DCRT

Project Description: Approximately 4000 patients have undergone operation for acquired and congenital heart disease in this Clinic. Pre- and postoperative hemodynamic, clinical assessment and long-term followup has generated valuable data concerning survival, complications, and functional improvement following operative intervention. Individuals who have undergone single or multiple valve replacement (n=1000) are being used as a pilot cohort of patients in this study. Survival, functional class improvement, hemodynamic results, specific incidences of emboli, prosthetic malfunction, infection, and reoperation are being analyzed. Autopsy findings or clinical cause of death is also being recorded. In addition to functioning as a bookkeeping device, the system also provides a more thorough followup system than currently exists. Whenever new data is acquired from a hospital admission, outpatient visit, or communication with the patient or their physician, the individuals file is updated. The data is also being transferred to microfiche for easy acquisition and storage.

Results: The 1000 series Starr-Edwards aortic valve patients (n=122) have been reviewed and entered into the program and results tabulated. Of the original 122 patients, 29 are known to be alive, 74 are dead and 19 have been lost to followup. The living patients now have a cumulative followup of 5042 months. The incidence of leading complications is: 1) thromboembolism 25%; 2) ball variance 23%; and 3) anemia 15%. Myocardial infarction and arrhythmia were the leading cause of death in this series. The 1200 (aortic) and 6000 (early mitral) series patients have been reviewed and entered into the computer program, but final analyses is not yet available. In addition all other valvular patients have been reviewed and currently are being entered into the program for print out and analyses.

Proposed Course: The utility of the system is being assessed with these valvular patients, and a decision will be made as to whether the system will be expanded to include other operated groups. The Cardiology Branch is considering a similar program for their unoperated patients. If such a system is adopted a complete natural history profile, with the operation being the variable, will be generated on each patient seen at the NHLI.

Keywords: Long-term valve followup, Computer valve followup

Project No. ZO1 HL 02603-01 SU
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Surgical management of acquired tricuspid valve disease

Previous Serial Number: None

Principal Investigators: Robert H. Breyer, M. D.
James McClenathan, M. D.

Other Investigators: Charles L. McIntosh, M. D, Ph.D.

Project Description: The management of tricuspid valve disease associated with mitral or mitral-aortic disease continues to be a controversial subject. In 1967 the argument for conservative management was presented by this Clinic. Since that time, our approach to tricuspid valve disease has become more aggressive and an increasing number of patients undergo tricuspid valve replacement or annuloplasty.

A retrospective review was undertaken to compare the results of conservative management vs. tricuspid annuloplasty vs. tricuspid valve replacement. During the years 1972-1974 seventy-six patients were diagnosed to have tricuspid regurgitation and/or stenosis by palpation of the valve at the time of operation. Twenty-one patients did not undergo any tricuspid procedure, 30 patients underwent tricuspid annuloplasties, 25 underwent tricuspid valve replacement with porcine xenografts.

Results: Operative mortality for the three respective groups was 10%, 23%, and 12%. By July 1, 1975 six month postoperative catheterization studies will be complete for all patients. At that time postoperative functional class and pre- and postoperative hemodynamics will be analyzed.

Preliminary data indicate that an aggressive surgical approach is indicated in the management of tricuspid valve disease. Tricuspid valve replacement is shown to be superior to tricuspid annuloplasty.

Proposed course: Upon completion of analyses of hemodynamic and clinical evaluation a paper will be submitted for publication.

Keywords: Tricuspid Valve Replacement, Tricuspid Annuloplasty.

Project No. Z01 HL 02604-02 SU
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Phonocardiographic detection of ball variance

Previous Serial No: NHLI-169

Principal Investigator: Lee C. Amsler, M. S.

Other Investigators: Harry W. Seipp
Robert Romanoff
Charles L. McIntosh, M. D.

Cooperating Unit: Division of Computer Research and Technology, Computer Systems Laboratory, NIH

Project Description: Phonocardiograms from patients with Starr-Edwards aortic prostheses, series 1000 and 1200, have been analyzed with the aid of a hybrid computer system. The following parameters are obtained from the phonocardiogram for evaluation: 1) a correlation coefficient (Rxy) for the opening prosthetic aortic valve sound, 2) a prosthetic aortic opening sound intensity to closing sound intensity ratio (AO/AC), and 3) a prosthetic aortic opening sound energy to closing sound energy ratio within a high frequencey window (Hi-Freq AO/AC). A spectrographic analysis was performed on a representative sample of our patient population to evaluate the use of this technique in the detection of silastic ball variance.

Results: Sixty-five silastic aortic valve patients, Starr-Edwards 1000 and 1200 series, have been studied via phonocardiography and fourteen patients with recorded phonocardiograms have come to reoperation or autopsy with findings of ball variance at the NIH since 1969. Twelve of these "variant" patients had indications of ball variance by one or more of the criteria being utilized. The remaining two "variant" patients were diagnosed pre-operatively via a percent stroke-length determination. Positive diagnoses of ball variance were obtained in 36% of the "variant" patients by the Rxy and in 72% of the "variant" patients by both the AO/AC ratio and Hi-Freq AO/AC ratio. The results from the spectral analysis evaluation supported the diagnostic use of prosthetic valve frequency patterns in the detection of ball variance.

Proposed Course: Phonocardiograms are routinely recorded on all Starr-Edwards aortic series valve patients and on all Starr-Edwards 6000, 6100, and 6120 series mitral valve patients in the NIH outpatient clinic. The functional status of the prosthetic valves in vivo are determined whenever possible in conjunction with the continual evaluation of the diagnostic techniques. Reports indicate that the spectrographic analysis technique is one of the most reliable and comprehensive methods for the evaluation of all

prosthetic heart valves. Our preliminary findings with this technique are very encouraging. We believe that a routine spectral analysis of phonocardiograms can be a valuable adjunct to attaining a more complete understanding of prosthetic valve dysfunction and the techniques currently employed for their detection.

Keywords: Phonocardiogram, prosthetic dysfunction, opening-closing ratio

Project No. Z01 HL 02605-07 SU
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: NU-5 atomic powered pacemaker - experimental and clinical evaluation

Previous Serial No. NHLI 49 (c)

Principal Investigator: Charles L. McIntosh, M. D.

Other Investigators: Peter Frommer, M. D.
Joseph E. Pierce, D.V.M.

Cooperating Units: Office of the Director and Section on Lab Animal Medicine & Surgery, NHLI

Project Description: Efforts began several years ago to develop a cardiac pacemaker energy source (Plutonium 238) suitable for human implantation. Since May 1969, sixty-five atomic powered pacemakers have been implanted in dogs for in vivo testing; of these, 30 are the currently used NU-5 series. The first human implant was done April 9, 1973 and the second implant in June of 1973. Thirty dogs were implanted with NU-5 series pacemakers between July and September 1972.

Results: One pacemaker in the 30 animals implanted with the NU-5 series pacemakers has intermittently failed and was removed for examination by Arco. A second long-term implant animal died suddenly, but no malfunction of the pacemaker was found. The remaining twenty-eight units are functioning well at this time. The first unit implanted in a human continues to function well. The second patient required two additional units because of persistent infection of the pacemaker sites. A third human implant was performed November 5, 1974. The three clinically implanted NU-5 series pacemaker continues to function well at this time.

Proposed Course: Animal evaluation will continue for an indefinite time period with those now implanted. Human implants will be evaluated over the next decade.

Keywords: Plutonium 238 pacemaker, long-life pacemaker

Project No. ZO1 HL 02606-06 SU
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Silastic ball variance detection

Previous Serial Number: NHLI-52 (c)

Principal Investigator: Charles L. McIntosh, M. D.

Other Investigators: William Schuette, B. S., E. E.
Andrew G. Morrow, M. D.

Cooperating Unit: Biomedical Engineering & Instrumentation Branch, NIH

Project Description: The technique for determining percent stroke-length of the poppet of a prosthetic valve (Starr-Edwards) was developed in 1970 at the NIH. Other techniques for diagnosing ball varinace have a low yield (approximately 25%) for detecting this possible fatal complication. The limiting factor in utilizing our technique is the ability to visualize the barium impregnated ball on cine.

Results: Fourteen patients have been studied and operated upon at the NIH for aortic ball variance since 1970. Twelve of these patients had other criteria of ball variance, but two patients were thought "normal" by existing criteria. The first patient required operation for a diseased mitral valve, and inspection revealed obvious ball variance of the aortic ball. The second patient was operated on for aortic ball variance determined by this technique and the poppet was found to be variant at the time of operation.

Proposed Course: This technique and the phonocardiographic analysis of all patients with silicone rubber poppets are being routinely screened for possible valve dysfunction or ball variance in our clinic. A cine camera is being installed in the Department of Radiology so that this study may be performed on routine outpatient visits. Plans are being made with Dr. Benedict Kinglsey of Hahnemann Medical College to organize a conference dealing with diagnosis of valve dysfunction to be held in the Spring of 1976.

Keywords: Ball variance, noninvasive technique, prosthetic dysfunction

Project No. Z01 HL 02607-01 SU
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Topical hypothermia system

Previous Serial Number: None

Principal Investigator: Charles L. McIntosh, M. D.

Cooperating Unit: Biomedical Engineering & Instrumentation Branch

Project Description: Topical hypothermia utilizing "4°" C iced saline has become an important adjuvant in myocardial preservation during open heart operations. The major disadvantages of iced saline are: 1) dilution of pump prime with saline; 2) difficulty maintaining desired 4° C temperature; 3) need for continuous suctioning of saline and; 4) cost of large volumes of sterile saline.

In an attempt to optimize the topical hypothermia concept and eliminate some of the above disadvantages, cooling coils of two sizes have been constructed. Each side of the coil is covered with a teflon shield to prevent direct myocardial contact. An ARTE-8 Nes labs refrigeration unit is used to cool and circulate 30% alcohol solution through the coil. The coil is placed in the posterior pericardial sack and enough sterile saline is added to cover the ventricles. The temperature of the saline is then controlled by a thermocouple sevomechanism to maintain the desired 4° C temperature.

A series of dogs being used for a hypothermia protection study have provided us with the experimental model to evaluate this system. Myocardial thermocouple probes have been used to compare myocardial temperature achieved with the conventional vs. this cooling system.

Results: Preliminary results show that we are able to obtain comparable myocardial cooling with both systems; but have eliminated the disadvantages of running iced saline.

Proposed Course: Further animal studies are being completed and plans are being made to utilize this system for topical hypothermia protection during cardiac operations. A paper will be submitted for publication when the system has been evaluated clinically.

Keywords: Topical hypothermia, myocardial preservation, cooling device

Project No. Z01 HL 02608-01 SU
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Symposium on Intraoperative Protection of the Myocardium

Previous Serial Number: None

Principal Investigators: Lawrence L. Michaelis, M.D.
Victor J. Ferrans, M.D.
Robert A. Guyton, M.D.
Paul R. Hickey, M.D.
Bruce A. Reitz, M.D.
William R. Brody, M.D.

Other Investigators: Charles L. McIntosh, M.D.
Andrew G. Morrow, M.D.

Cooperating Unit: Section on Pathology, NHLI

Project Description: On June 11 and 12, 1974, the Clinic of Surgery of the NHLI hosted a symposium on Intraoperative Protection of the Myocardium. The symposium was sponsored and funded by the John E. Fogarty International Center for Advanced Study in the Health Sciences and participants included the intramural investigators listed above as well as numerous scientists from outside the NIH.

The symposium was organized as a forum for surgical investigators to become familiar with each others work and to discuss their goals and techniques with investigators of different disciplines who are interested in this general area. Participants representing the fields of physiology, pharmacology, biochemistry, and pathology were included in the symposium.

Results and Proposed Course: The proceedings of the symposium have been transcribed and edited and will be published in a special edition of The Annals of Thoracic Surgery in July, 1975.

Keywords: Myocardial Protection, Cardiopulmonary Bypass, Cardiovascular Surgery

Project No. Z 01 HL 02609-02 SU
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The effect of dipyridamole and methylprednisolone on intimal proliferation in venous autografts used for arterial bypass

Previous Serial Number: NHLI-191

Principal Investigators: William R. Brody, M.D.
John W. Brown, M.D.
Bruce A. Reitz, M.D.
Paul R. Hickey, M.D.

Other Investigators: Donald L. Fry, M.D.
Lawrence L. Michaelis, M.D.

Cooperating Unit: Section on Experimental Atherosclerosis, NHLI

Project Description: Eighteen dogs underwent 36 femoral artery bypass procedures using autologous femoral vein and were placed into three groups; no treatment; postoperative treatment with dipyridamole; and postoperative treatment with methylprednisolone. After six weeks, femoral angiography was performed to assess graft patency, the animals were killed, and the grafts removed and prepared for histologic examination.

Results: Overall graft patency was 94 percent, with no significant differences among groups. Mean intimal thickness measurements were taken in the proximal, middle, and distal tissues of the grafts to quantitate intimal proliferation. The differences between control and dipyridamole treated groups were not significant. Dogs treated with methylprednisolone had significantly less intimal thickening in the middle third than controls (125 microns vs. 214).

These data show no detectable response of intimal proliferation to dipyridamole therapy. While corticosteroids effectively decreased thickening in the middle of the vein grafts, substantial thickening remained near the anastomotic sites, suggesting that factors relating to blood flow and operative technique are of importance.

Proposed Course: These results have been submitted in abstract form for presentation to the Surgical Forum of the American College of Surgeons.

Keywords: Intimal Proliferation, Coronary Bypass Grafts, Dipyridamole, Methylprednisolone

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Protection of the myocardium during anoxic arrest

Previous Serial Number: NHLI-189

Principal Investigators: William R. Brody, M.D.
Bruce A. Reitz, M.D.
Paul R. Hickey, M.D.
Michael J. Andrews, M.D.
Lawrence L. Michaelis, M.D.

Other Investigator: William C. Roberts, M.D.

Cooperating Unit: Section on Pathology, NHLI

Project Description: Numerous studies have shown acute alterations in left ventricular function and morphology after cardiopulmonary bypass (CPB), but long-term changes occurring in chronic survivors have not been documented. This study assesses the long-term effects of CPB in combination with popular methods of myocardial protection.

Thirty-seven dogs were placed on CPB for 100 minutes using a bubble oxygenator without hemodilution, maintaining mean aortic pressure at 80 mm. Hg. The left ventricle was vented. The animals were divided into three groups:

I. Normothermic anoxic arrest for 60 minutes (aortic occlusion).

II. Continuous coronary perfusion with an empty, beating heart for 60 min. at 35° C.

III. Hypothermic anoxic arrest (aortic occlusion) for 60 min. with topical cold saline lavage (4° C.).

Survival in Groups II and III was significantly better than Group I (82% and 92% versus 45%). The 22 survivors of all groups underwent left heart catheterization and LV cineangiography five months later, and were compared to a control group of eight normal dogs.

Results: All three groups had significant elevation of LVEDP ($p < .01$), depression of maximum dp/dt ($p < .05$), and signs of LV dysfunction on angiography when compared to the normal dogs. Significant differences in function between Groups II and III were not observed. Gross subendocardial fibrosis was present in the hearts of all survivors. The infusion of solutions similar to extracellular fluid (Ringers solution), or intracellular

fluid (Sacks solution) during anoxic arrest in Groups I and III did not affect survival or chronic function.

These data indicate that although survival is greatly enhanced when coronary artery perfusion or topical hypothermia is used, neither method prevents chronic deterioration in ventricular function nor the development of subendocardial fibrosis.

Proposed Course: This study will be presented at the first meeting of the Samson Thoracic Surgical Society in May, 1975 and will be published in the Journal of Thoracic and Cardiovascular Surgery. This paper has been awarded the Prize Manuscript in the Intern/Resident Division of the Samson Thoracic Surgical Society.

Keywords: Cardiopulmonary Bypass, Myocardial Protection, Subendocardial Fibrosis

Project No. Z01 HL 02611-01 SU
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The contribution of atrial contraction to right heart function before and after right ventriculotomy: Experimental and clinical observations

Previous Serial Number: None

Principal Investigators: Robert A. Guyton, M.D.
Michael J. Andrews, M.D.
Paul R. Hickey, M.D.

Other Investigators: Lawrence L. Michaelis, M.D.
Andrew G. Morrow, M.D.

Project Description: The effects of loss of effective atrial contraction and of right ventriculotomy upon right heart function were studied in a hemodynamically controlled canine preparation. A clinical study was then undertaken of the effects of loss of effective atrial contraction in two groups of postoperative patients, one group with right ventriculotomy and a control group without ventriculotomy.

Results: In open-chest dogs, loss of effective atrial contraction had little effect upon right heart function. Similarly, ventriculotomy alone had little deleterious effect, but loss of atrial contraction after ventriculotomy caused a marked elevation in right atrial pressure (9.5 mg. Hg, $p < .01$) at a constant cardiac output, aortic pressure and heart rate. Restoration of atrial contraction led to a dramatic reversal of volume-induced right heart failure (right atrial pressure of 22 mm. Hg decreased to 4 mm. Hg, $p < .001$) after ventriculotomy.

In eight patients who had had right ventriculotomies, abolition of effective atrial contraction caused an average reduction in cardiac output of 22%, whereas cardiac output fell only 5% in five control patients ($p < .01$).

Either ventriculotomy or loss of effective atrial contraction is well tolerated by the right heart, but the combination of ventriculotomy and loss of atrial contraction may cause clinically significant impairment of right heart function.

Proposed Course: This study will be presented at the Annual Meeting of the American Association for Thoracic Surgery in April 1975, and will be submitted for publication to the Journal of Thoracic and Cardiovascular Surgery.

Keywords: Atrial Contraction, Right Heart Function, Ventriculotomy

Project No. Z01 HL 02612-01 SU
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: A mechanical device for sutureless aortosaphenous vein anastomosis

Previous Serial Number: None

Principal Investigators: Robert A. Guyton, M.D.
Michael J. Andrews, M.D.
James H. McClenathan, M.D.

Other Investigator: Lawrence L. Michaelis, M.D.

Project Description: Previously developed techniques for sutureless vascular anastomosis have required eversion of both the distal and proximal vessel. Since the human aorta, particularly when it is pathologically thickened, is not easily everted, a mechanical device has been developed for sutureless anastomosis of a vein segment to a non-everted aorta. This device was used in twenty dogs for chronic interposition of a reversed autologous vein segment between the aorta and the distal portion of the divided left subclavian artery.

Results: Angiographic examination two to three weeks after operation revealed that 17 of 20 grafts were patent. Four of these 17 animals were killed at one month after operation and four were killed three months after operation. All eight autopsy specimens revealed widely patent anastomosis with an intact endothelial covering over the mechanical device. Corrosion of portions of the stainless steel device was evident.

Proposed Course: Data from this study will be compiled and presented for publication to report the feasibility of this type of anastomosis. The surviving animals will be killed one year after operation for pathologic study.

Keywords: Sutureless Anastomosis

Project No. Z01 HL 02613-01 SU
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Subendocardial ischemia during partial coronary occlusion in dogs: The significance of S-T segment elevation in subendocardial electrograms

Previous Serial Numbers: None

Principal Investigators: Robert A. Guyton, M.D.
Michael J. Andrews, M.D.
James H. McClenathan, M.D.
Glenn E. Newman, M.D.

Other Investigators: Victor J. Ferrans, M.D.
Lawrence L. Michaelis, M.D.

Cooperating Unit: Section of Pathology, NHLI

Project Description: Partial coronary occlusion was evaluated in open chest animals by recording epicardial and endocardial electrograms from the area of distribution of the occluded artery. Radioactive microsphere measurements of regional coronary flow were made before, during, and two to four minutes after partial coronary occlusion. Finally, animals subjected to three hours of partial coronary occlusion were killed two weeks later for pathological examination.

Results: As partial coronary occlusion is gradually increased, endocardial S-T segment elevation occurs at a higher distal coronary pressure than does epicardial S-T segment elevation. Coronary occlusion was adjusted to effect endocardial without epicardial S-T elevation at a distal coronary pressure of 40-50 mm. Hg. Microsphere measurements of regional coronary flow in this situation revealed no change in epicardial flow (.90 to .99 cc/min/gm) and a 76% reduction in endocardial flow (1.00 to .24 cc/min/gm, $p < .001$). Measurement of regional myocardial flow at 2 to 4 minutes after release of the occlusion demonstrated no change from pre-occlusion levels in epicardial flow (.97 to 1.02 cc/min/gm) and reactive hyperemia in the subendocardial layer (1.02 to 2.80 cc/min/gm, $p < .01$). Endocardial without epicardial S-T segment elevation was maintained for three hours in a second group of dogs. A full-thickness myocardial biopsy was taken for electron microscopic examination at the end of the three hour period. Results of electron microscopy are pending. Pathologic examination of these animals two weeks after operation revealed focal subendocardial infarction in all animals without full-thickness infarction. It is concluded that endocardial S-T segment elevation is associated with subendocardial ischemia and infarction and that epicardial S-T segment elevation may be absent during subendocardial ischemia which is sufficiently severe to cause infarction.



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July 1, 1974 through June 30, 1975

Proposed Course: After histologic evaluation is completed, this study will be submitted for publication.

Keywords: Subendocardial Ischemia, Endocardial Electrograms, Regional Coronary Flow, Partial Coronary Occlusion

Project No. Z01 HL 02614-01 SU
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Subepicardial and subendocardial ischemia following coronary occlusion

Previous Serial Number: NHLI-193

Principal Investigators: Robert A. Guyton, M.D.
Michael J. Andrews, M.D.

Other Investigator: Lawrence L. Michaelis, M.D.

Project Description: Epicardial and endocardial electrodes have been used to record S-T segment elevation in the area of an acute myocardial infarction in open chest dogs, with careful control of hemodynamic variables. Five minute occlusions of the distal left anterior descending coronary artery under constant hemodynamic conditions caused reproducible epicardial and endocardial S-T segment elevation.

Results: The endocardial and epicardial S-T segment elevation caused by a five minute coronary occlusion is increased by increasing cardiac output, increasing heart rate or decreasing aortic pressure when other hemodynamic variables remain constant ($p < .05$). Elevation of aortic pressure caused a greater decrease in epicardial S-T elevation than in endocardial S-T elevation ($p < .05$) while changes in cardiac output or heart rate caused alterations in epicardial S-T elevation which were quantitatively similar to changes in endocardial elevation. Nitroglycerin infusion in these animals, at a constant heart rate, aortic pressure, and cardiac output caused no change in endocardial or epicardial S-T segment elevation with a five minute coronary occlusion.

Proposed Course: Preliminary results of this study were presented in a Symposium on Intraoperative Protection of the Myocardium to be published in the July issue of Annals of Thoracic Surgery. Final results will be compiled and submitted for publication.

Keywords: Coronary Artery Occlusion, Subendocardial Ischemia, Endocardial and Epicardial Electrograms

Project No. Z01 HL 02615-01 SU
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Alterations in regional ischemia following partial coronary occlusion: The effects of changes in blood pressure, heart rate, and cardiac output

Previous Serial Number: None

Principal Investigator: Robert A. Guyton, M.D.

Other Investigators: Lawrence L. Michaelis, M.D.

Project Description: In an open chest canine preparation, endocardial and epicardial S-T segment elevations were recorded as circumflex coronary pressure was decreased (in steps of 10 mm. Hg) at a constant aortic pressure, heart rate and cardiac output. Recordings were then repeated at a different aortic pressure, heart rate, or cardiac output.

Results: As circumflex coronary pressure is decreased endocardial S-T segment elevation gradually increases as coronary pressure is decreased from 70 to 50 mm. Hg and generally fails to increase further. Epicardial S-T segments, however, are depressed as coronary pressure is decreased from 70 to 50 and then rise rapidly as coronary pressure is decreased further. Preliminary results indicate that increases in aortic pressure, heart rate, or cardiac output tend to increase the level of endocardial S-T segment elevation present at any circumflex coronary artery pressure and tend to increase the pressure at which epicardial S-T segment elevation occurs.

Proposed Course: Additional experiments are necessary to provide sufficient data for statistical analysis. Further studies may be conducted upon the effects of nitroglycerin during partial coronary occlusion with constant aortic pressure, heart rate, and cardiac output.

Keywords: Partial Coronary Occlusion, Regional Coronary Ischemia, Blood Pressure, Heart Rate, Cardiac Output

Project No. Z01 HL 02616-01 SU
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Temporal changes in regional coronary flow after partial coronary occlusion

Previous Serial Number: None

Principal Investigators: Robert A. Guyton, M.D.
James H. McClenathan, M.D.

Other Investigators: Lawrence L. Michaelis, M.D.

Cooperating Units: Radiation Safety Office

Project Description: Regional myocardial blood flow was measured using radioactive microspheres during partial coronary occlusion in dogs. Measurements were made before, during, and after a variable occlusion which maintained a constant distal coronary pressure of 40 to 45 mm. Hg. A second group of dogs was studied at 5, 30, 90, and 180 minutes after an occlusion which maintained a constant proximal circumflex coronary artery resistance for three hours.

Results: In five dogs at a constant distal circumflex coronary artery pressure, regional coronary flow was unchanged over a three hour period in the unoccluded area (.76 to .71 cc/min/gm) and in the epicardial layer of the occluded area (.56 to .48 cc/min/gm) but fell by more than 50% in the endocardial layer of the occluded area (.22 to .09 cc/min/gm, $p < .02$). Resistance to coronary flow increased in the ischemic endocardial layer by more than 100%, suggesting that ischemia may be self-propagating. Release of the partial occlusion led to full restoration of flow in all layers of the occluded area.

A second group of twelve dogs was studied during a three hour occlusion simulating a constant proximal coronary resistance. Distal coronary pressure and flow to the occluded area increased in all 12 dogs during the first 30 minutes of occlusion, but changes at 90 and 180 minutes varied from dog to dog. In seven animals in which maximum flow to the endocardial layer of the occluded area was less than 0.7 cc/min/gm, changes in distal coronary pressure and in flow to the occluded area were linearly related ($r = .81$, $p < .001$ and $r = .80$, $p < .001$) to directionally opposite changes in flow to the nonoccluded area. This result suggests that a coronary steal phenomenon may be operative in these animals. In the five animals with maximum endocardial flow greater than 0.7 cc/gm/min, no relationship between flow to the occluded area and flow to the nonoccluded area was observed. In all 12 dogs, flow to the endocardial layer of the occluded area was directly proportional to distal coronary pressure ($r = .93$, $p < .001$), but epicardial flow tended

not to decrease until coronary pressure fell below about 40 mm. Hg.

Results: This study has identified two positive feedback systems, or "vicious cycles", which may be important in the temporal evolution of myocardial infarction: 1) A time-related increase in coronary resistance in ischemic areas and 2) decrease in flow to an ischemic area as flow to adjacent nonischemic areas is increased.

Proposed Course: This study is complete and a manuscript is in preparation.

Keywords: Regional Coronary Flow, Partial Coronary Occlusion, Coronary Resistance, Subendocardial Ischemia

Project No. Z01 HL 02617-01 SU
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Extended storage of the canine heart for transplantation

Previous Serial Number: None

Principal Investigators: Donald C. Watson, M.D.
David L. Gregg, M.D.

Other Investigator: Charles L. McIntosh, M.D., Ph.D.

Project description: Canine hearts are removed from donor dogs and perfused for 48 hours in a specially constructed chamber with a solution which is free of calcium and saline and rich in potassium, glucose, and oxygen. The perfusion pressure is constant at 18 mm. Hg. Coronary flow, fluid pH and electrolyte concentrations are monitored. At 48 hours of perfusion graft viability is tested by orthotopic homotransplantation into a suitable recipient.

Results: Most of the hearts of recent experiments have been able to maintain the circulation of the recipient for 4-72 hours without inotropic support. The graft viability has been directly related to the amount of coronary flow during the perfusion. The determinants of coronary flow are established at the initial arrest and are currently under investigation.

Proposed Course: Various methods of elective cardioplegia are being tested and their effects on coronary resistance measured. The effects of coronary vasodilators are also being ascertained. Once low coronary resistance has been established, cardiac preservation for periods longer than 48 hours will be attempted.

Keywords: Cardiac Transplantation, Myocardial preservation

Project No. Z01 HL 02618-01 SU
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Anatomic correction of transposition of the great vessels

Previous Serial Number: None

Principal Investigator: David L. Gregg, M.D.

Other Investigator: Charles L. McIntosh, M.D., Ph.D.

Project Description: Under total cardiopulmonary bypass, a model of transposition of the great vessels is made in dogs by the construction of an intra-atrial baffle. The transposition complex is then corrected at the great vessel level by constructing a truncus arteriosus utilizing the proximal segments of the aorta and pulmonary artery and distal aorta. The pulmonary blood flow is then established between the left ventricle and distal pulmonary artery by means of an extra cardiac shunt.

Results: Of 15 dogs, only one has been successfully separated from cardiopulmonary bypass; the remaining dogs dying from acute right ventricular failure.

Proposed Course: Currently a population of puppies who have undergone pulmonary artery banding are growing into adult size. At that time further attempts will be made to establish this correction in the hope that the developed right ventricular hypertrophy will enable the right ventricle to sustain systemic work.

Keywords: Transposition of Great Vessels, Operative Procedure

Project No. Z01 HL 02619-01 SU
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Study of the rate of development and severity of arterial atheromas in high and normal flow states of hypercholesterolemic animals

Principal Investigators: Michael S. Cann, M.D.
Robert J. Breyer, M.D.

Other Investigators: Charles L. McIntosh, M.D., Ph.D.
Donald Fry, M.D.
Donald Watson, M.D.

Cooperating Unit: Section of Experimental Atherosclerosis, NHLI

Project Description: Three groups of adult mongrel dogs were prepared in the following manner: the first underwent creation of unilateral carotid-jugular and femoral-femoral arterio-venous fistulae, followed by total thyroidectomy and ingestion of a high cholesterol diet; the second underwent simultaneous shunting and thyroidectomy followed by the special diet; the final group had thyroidectomy and special diet followed by shunting at a later date. All animals were carried on a high cholesterol diet for six months and followed with weekly or biweekly serum cholesterol and thyroid function studies.

Results: None of the animals has been sacrificed, but all will have been within the next two months.

Proposed course: Animals will be sacrificed after six months of special diet, and shunted vessels as well as contralateral normal vessels removed for gross and microscopic study.

Keywords: Arterio-venous fistulae, Thyroidectomy.

Project No. Z01 HL 02620-01 SU
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The effects of isoproterenol and dopamine on regional coronary flow distribution in dogs with partial coronary occlusion

Previous Serial Number: None

Principal Investigators: James H. McClenathan, M.D.
Robert A. Guyton, M.D.

Other Investigator: Lawrence L. Michaelis, M.D.

Project Description: In 11 dogs, partial occlusion of the circumflex coronary artery was created. Left atrial, aortic, and distal circumflex coronary artery pressure, cardiac output, heart rate and left ventricular dp/dt were monitored. Pharmacologic doses of isoproterenol and dopamine were given. Regional coronary flow was measured with radioactive microspheres.

Results: Dopamine caused an increase in aortic and distal circumflex coronary artery pressure, cardiac output and peak dp/dt. There was no redistribution of regional coronary flow.

Isoproterenol caused an increase in heart rate, cardiac output and peak dp/dt, while aortic and distal coronary pressure decreased. Total flow to the myocardium distal to the occlusion was unchanged. However, there was redistribution of flow away from the subendocardium ($p < .01$). We are proposing that the redistribution of coronary flow distal to the occlusion which follows administration of isoproterenol is related to the decrease in perfusion pressure and the increase in myocardial contractility.

Proposed Course: A manuscript is in preparation.

Keywords: Regional Coronary Flow, Partial Coronary Occlusion, Dopamine, Isoproterenol

Project No. Z01 HL 02621-01 SU
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Distribution of blood flow before and after repair of coarctation of aorta

Previous Serial Number: None

Principal Investigator: Glenn E. Newman, M.D.
Michael J. Andrews, M.D.

Other Investigators: Lawrence L. Michaelis, M.D.

Project Description: Repair of aortic coarctation is sometimes associated with two hazardous postoperative complications; a severe exacerbation of the pre-existing systemic hypertension and mesenteric vasculitis. A sudden change in pressure and flow distal to the coarctation has been suggested as the initiator of these postoperative events, but laboratory assessment of the clinical situation has been hampered by the difficulty in creating an appropriate laboratory model and by accurate methods of measuring flow to various organs over a several day period.

We have developed a successful method of producing aortic coarctation in young dogs by constructing the descending thoracic aorta with a tape tied over felt pads. After these animals have matured we will repair the coarctation and will perform chronic flow measurements by injecting radioactive microspheres via a catheter implanted in the left atrium. Flow to the myocardium, gut, kidney, and skeletal muscle will be assessed in these animals (unanesthetized) prior to repair, in the immediate postoperative period, and after postoperative equilibration occurs. Chronic aortic pressure measurements will be made proximal and distal to the coarctation.

Results: Retrograde catheterization has demonstrated a significant coarctation in these animals (six weeks after operation - average gradient 55 mm. Hg).

Proposed Course: After the animals have reached a stable condition following coarctation repair (10-14 days), they will be killed and the above mentioned organs will be analyzed for the various microspheres injected before and after the operation. Thus, flow to these organs before and after repair can be quantitated. It is hoped that this information, pressure changes that occur, and renin and angiotension assays in these animals will help identify the etiology of these potentially lethal postoperative events.

Keywords: Coarctation of Aorta, Repair, Distribution of Blood Flow

Project No. Z01 HL 02622-01 SU
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 thorugh June 30, 1975

Project Title: Study of arterial wall permeability as a function of flow rate

Previous Serial No:

Principal Investigators: Lynn H. Harrison, M. D.
Michael S. Cann, M. D.

Other Investigators: Charles L. McIntosh, M. D.
Donald L. Fry, M. D.

Cooperating Unit: Section of Experimental Atherosclerosis, NHLI

Project Description: Adult mongrel dogs were subjected to creation of ipsilateral carotid-jugular and femoral-femoral arteriovenous fistulas as well as corresponding arteriotomies on the contralateral side. Animals were then sacrificed at 24, 48, and 72 hours as well as at 4 weeks to one year following shunting. All were given an intravenous dose of Evan's Blue Dye, U.S.P., 1.5 cc/Kg, 24 hours prior to sacrifice. At sacrifice, vessels were harvested on both shunted and nonshunted sides and scanned with a photosensitive cell capable of detecting Evan's Blue Dye concentration. Microscopic sections of vessels were also taken.

Results: Arterial wall permeability appeared to be greatest during the first 24-48 hours following shunting, and tapered off exponentially in the ensuing days and weeks. Preliminary microscopic examination suggests a thickening of intima and possibly media in shunted arteries as well as a realignment of intimal cells in response to increased flow.

Proposed Course: Several additional animals have been shunted to fill out groups of long-term animals in order to make the sample group large enough for statistical comparison with the other groups. These animals await sacrifice at the appropriate intervals.

Keywords: arterial wall permeability

ANNUAL REPORT OF THE
SECTION ON EXPERIMENTAL ATHEROSCLEROSIS
NATIONAL HEART AND LUNG INSTITUTE
July 1, 1974 through June 30, 1975

The program for the Section on Experimental Atherosclerosis has continued to be targeted on the mechanisms leading to the genesis of the atherosclerotic plaque, the lethal lesion of atherosclerosis. The ultimate aim of this program is to identify each of the steps in the evolutionary sequence of this disease process in terms of cellular metabolic mechanisms and physical chemical laws so that deliberate strategies can be developed to interupt this sequence at various selected points of vulnerability. This broad objective has required the development of a multidisciplinary program consisting of experimental pathology, human pathology, lipoprotein chemistry, physical chemistry, arterial tissue culture, vascular physiology and data processing. The present report deals only with achievements in our most active areas this year and, therefore, a brief review of certain background information and the overall program plan will help the reader to place current efforts in clearer perspective.

Background Information and Overall Program Plan

A variety of experimental observations from this section, which have been covered in previous annual reports, are consistent with the following working hypothesis upon which the program of this section is based. We have shown that macromolecular (albumin) transport into the vascular interface is not uniform along the arterial tree but tends to vary with a typical topographic pattern in normal animals. This pattern appears qualitatively identical to the pattern of intimal lipid deposition (sudanophilia) that develops in the same species when serum cholesterol is elevated by dietary means. This set of observations can be explained by the following hypothetical sequence.

Endothelial permeability is altered by a local hemodynamic or structural factors such that there is a greater flux of plasma substances across the endothelial surface into the intimal tissues at these sites. This increased flux results in increased tissue concentrations of certain of the plasma substances. The concentration profile across the wall will depend not only on the magnitude of the flux but also upon the locations and nature of the barriers to this flux. Generally speaking, the concentration will be highest in the intimal region, will decrease across the wall, and become minimal in the lymphatic spaces of the adventitia. Smooth muscle cells have been shown to proliferate in the presence of increased concentration of plasma substances. These cells metabolize the lipoproteins by hydrolizing the triglyceride present. However, they cannot metabolize the associated cholesterol in the lipoproteins and therefore these cholesterol substances tend to accumulate in the form of intracellular inclusions and extracellular aggregates. These aggregates of cholesterol products apparently cannot be resolublized and transported out of the vascular tissue rapidly enough to prevent their progressive accumulation. The tissue responds to the accumulation as to any other "foreign" body, ultimately walling it off with fibrous tissue and chronic inflamatory cells thereby producing the mature atheromatous plaque.



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In accordance with the above scheme, the program has been structured around study of the atherogenic precursors, study of the transport processes by which these precursors are carried into the intimal space, and studies of the various tissue responses to the presence of these atherogenic precursors. Progress in these three important areas over the past year are outlined below.

Tissue Responses

During the past year we have continued to examine the response of arterial tissue components to increase concentration of plasma substances. These studies have been carried out in tissue culture as well as in acute and chronic studies in normal and hyperlipidemic animals.

The removal of the endothelial surface in normal animals using an intravascular balloon technique has been shown to result in a greatly increased flux of plasma substances into the eroded intimal surface which is followed by an enormous proliferative response of the smooth muscle cells at the vascular interface. This proliferative response continues until the surface becomes covered again with a normal endothelial cell layer which may take from weeks to months. With the reappearance of the endothelial cell layer, the increased transport of plasma substances into the intimal space is returned to normal and the smooth muscle cell proliferative response subsides, resulting in a residual intimal thickening indistinguishable from that seen in older animals. The same studies carried out in hyperlipidemic animals are also associated with a proliferative response of the smooth muscle cells, however, with marked intracellular and extracellular lipid deposition resulting in an atheromatous lesion.

These observations are consistent with the tissue culture work reported in last year's annual report and which have shown that smooth muscle cells in tissue culture proliferate rapidly in the presence of the low density lipoproteins and become laden with intracellular lipid vacuoles particularly when exposed to VLDL. The above observations suggest that the initial lesion of atherosclerosis is a proliferative response of arterial smooth muscle cells when they are exposed to an increase in the tissue concentration of certain plasma substances, e.g., lipoproteins.

Further insights into tissue responses were gained from study of our animal colonies. Three important animal colonies were harvested and revealed some new and interesting results. The first of these was the primate colony (Erythrocebus Patas monkeys) which was studied in collaboration with the Veterinary Resources Branch in DRS. The primates were studied in three groups: one on normal diet, one on a high fat diet, and one on a high fat diet to which one-half of one percent cholesterol was added. The control animals developed insignificant disease, whereas the high fat animals developed occasional fatty streaking and, only rarely, developed plaques. In sharp contrast to these, the cholesterol fed animals developed florid proliferative atherosclerosis progressing from fatty streaks to raised plaques and finally complicated plaques with occasional thrombosis.

The second study, which was carried out under contract with the University of Missouri, was designed to examine the influence of age, sex and diet on the arterial tissue changes in miniature swine. Histologic examination of these

tissues revealed a histologic pattern similar to that seen in the monkeys, but differed in that the lesions were slightly more fibrotic and did not show any evidences of thrombotic activity. The severity of the disease both in the monkey and swine colonies appeared to correlate principally with the level of serum cholesterol and not with other variables such as age or sex. The disease in both species was principally intimal with only moderate, secondary medial changes and was in every respect indistinguishable from the human counterpart.

The third colony was composed of NIH foxhounds which were studied under contract to the Colorado State University with a protocol similar to the above swine study, i.e., to determine the effect of age, sex, and dietary cholesterol on the development of atherosclerosis. The histologic characteristics of the disease produced in these animals was different from that seen in the above primate and swine studies as well as different from that seen in previous studies with foxhounds carried out at NIH. For a given serum cholesterol level the lesions produced in these animals were much more severe both in extent and by histologic criteria than were the lesions examined in the previous studies. There was a marked tissue destruction with invasion of histiocytes and polymorphonuclear leukocytes. Spread of the process from the intima to involve the entire media of the vessel was common at many locations. There was frequent evidence of prior thrombosis with recanalization of the vessels. Seven clinically significant thrombotic accidents occurred in this colony, including myocardial infarcation and gangrene of an extremity. Moreover, the overall topographic distribution of the lesions was more peripheral and less central than in the previous studies in the foxhounds as well as in the swine and primate studies.

The principal difference in the experimental protocol governing this experimental colony was the inclusion of beef tallow and taurocholic acid (a naturally occurring bile salt) in the diet of these animals. We conclude tentatively from these important observations that while the serum cholesterol appears to be a common governing variable in this disease process, other factors such as bile acid metabolism and the composition of the triglyceride appear to be important modulating influences on the tissue response to the increase tissue concentration of the atherogenic precursors.

Study of the Atherogenic Precursors

Evidence has mounted from our studies this year that the atherogenic precursors in all species are closely related to the cholesterol carried in the low density serum lipoproteins. Two sorts of studies support this. The first are the colony studies which, as indicated above, continue to show a strongly positive correlation between the serum cholesterol level in a given species and both the extent and severity of the associated atherosclerotic processes. The second are the detail studies of the changes in the lipoproteins that occur concomitant with the phase of the rapid progression of the atherosclerotic process. Detailed analysis of the plasma lipoproteins indicates that dietary cholesterol induces a characteristic hyperlipoproteinemia with a lipoprotein pattern consistent among the various species. The rapid proliferative phase of atherosclerosis is associated with an increased cholesterol and cholesterol ester content of several different lipoproteins in the low

and very low density fractions. Analysis of the apoprotein content of these lipoproteins has revealed an important new correlation, i.e., the appearance of a characteristic arginine-rich apoprotein. This apoprotein has been found in all species that have been examined (monkey, pig, dog, rabbit, rat and type III human hyperlipoprotenemia) and may function in the transport of cholesterol between lipoproteins and the arterial wall. Thus, it appears that an atherogenic factor is associated in all species with the appearance of certain cholesterol carrying lipoproteins that have a common arginine-rich apoprotein. The structural characteristics, physical properties, and metabolism of these lipoproteins are currently under intensive study in this laboratory.

Studies of Transvascular Macromolecular Transport

It is highly probable that the atherogenic precursors are either macromolecules or are carried on macromolecular vehicles from the blood into the intimal space. As discussed above, we are hopeful that we are converging on the chemical identity of these and,in anticipation of this, have spent considerable effort this year developing the necessary technical, physical chemical, and mathematical tools to quantify these important transport processes. The serum albumin and the Evans blue dye albumin complex transport systems have been developed as prototype models for this purpose to devise the necessary theory, experimental, and analytic protocols for the future studies.

Experiments were carried out using a specially devised organ support system in which it was possible to study the freshly excised aorta under controlled conditions of mechanical constraint, temperature, bathing milieu, and transmural pressure. Using iodine tagged albumin the following observations were made. Stirring of the albumin solution over the vascular interface does not appreciably increase the uptake of albumin. From this it was concluded that a significant diffusion barrier does not normally exist at the vascular interface and, therefore, transport properties are determined only by the wall. The second set of observations demonstrated that the accumulation of tagged albumin in the intimal space varied as the square root of time. This observation plus corroborative measurements of the concentration distribution across the wall (using autoradioagraphic techniques) indicated that the albumin transport system was following the mathematical solution of Fick's second law of diffusion as applied to diffusion in a two-phase system. Studies using doubly tagged albumin (covalently tagged with iodine and "complexed" with Evans blue dye) indicated that the accumulation of Evans blue dye in the intimal space was approximately four times greater than the accumulation of tagged albumin. Moreover, studies of the rate of elution of Evans blue dye back out of the intimal space indicated that the elution rate into a saline milieu was less than one-tenth of that into an albumin-saline milieu.

We conclude from these two sets of studies that albumin is transported into the intimal space in accordance with classic diffusion theory and that Evans blue dye is transported into and through the intimal space on albumin as a vehicle. However, in the intimal space the Evans blue dye enters into a new competitive equilibrium between tissue albumin and some fixed, binding substance in the wall. Various binding relationships were explored and it was

found that a simple linear adsorption isotherm described the binding process adequately. Measurements of the partition coefficient for albumin between blood and the tissue space were carried out using iodine tagged albumin and measuring the plateau on the uptake curve for the tissue. This plateau occurred at about fifteen hours and the value of the partition coefficient was approximately .20, that is to say, the equilibrium volume-concentration of albumin in the tissue space is about one-fifth of that in the blood. The albumin uptake curves were then evaluated using this partition coefficient to determine the diffusion coefficient of albumin in arterial tissue. This was found to be approximately $2 \times 10^{-8}$ square centimeters per second. With this information it was possible then by comparison of Evans blue dye uptake and the tagged albumin uptake to calculate the binding constant for Evans blue dye in the wall. This was found to be about 15, i.e., the amount of Evans blue dye bound to structural substances in the wall is about 15 times that free to diffuse on tissue albumin. Thus, it has been possible to define the diffusion of albumin as well as the diffusion and chemical complexing of a substance carried on albumin in rather complete and unambiguous physical chemical terms. This represents a significant advance in a previously obscure area of transport mechanics.

The transmural pressure acts as an added driving force for this macromolecular flux. The magnitude of this was evaluated by creating a transmural pressure of 100 mm of mercury and measuring the resultant increment increase in flux. This increment increase represented the "convective flux" related directly to the imposed transmural pressure. Under steady state conditions, this was found to be approximately one-half of the diffusive flux across the wall. (In calculating the steady state diffusive flux values from the literature were used for the concentration of the diffusing species in the adventitial lymphatic space.)

Normally, the principal barrier to transport resides in the endothelial surface itself. As indicated earlier, we have shown that transmural transport increases enormously in regions of endothelial injury and even under normal conditions is also increased at certain characteristic sites along the arterial tree.

Detailed electron microscopic studies are being carried out to discover the ultrastructural features of endothelial cells that might correlate with increased transendothelial transport. When viewed by scanning electron microscopy, the endothelial surface in areas of increased transport is more disordered, has poorly defined cell borders and nuclei, and appears to have a "soft" or "boggy" texture. Transmission electron microscopy shows surprisingly little evidence of abnormal endothelial structure but does show moderate subendothelial edema in these regions. Although the above are by far the most common morphologic features of regions of increased transport, discrete areas of endothelial cell erosion are also observed occasionally, suggesting that these areas may be populated by a more fragile cell surface or that these areas are episodically exposed to abnormally high hemodynamic forces.

Methods have been devised to measure the discrete rheologic properties of these areas using small calibrated jets of fluid to establish the cell "yield stress" and using a device with a small indenting probe to establish subsurface

"hardness." Although the jet studies are still in the developmental stage, preliminary data from the indentation studies suggest that areas of increased transport are "softer." Hemodynamic studies (detailed in previous reports) have been carried out in a continuing effort to define the detailed stress pattern to which this fragile surface is exposed.

In conclusion, it has been possible to define certain fundamental transport forces and the associated fluxes for albumin and albumin Evans blue dye complex across the intimal-medial space. These relationships have been shown to follow classical non-equilibrium thermodynamic relationships thereby establishing the fundamental physical chemical behavior of the albumin transport system into the intima and media. With these prerequisite determinations accomplished, we are now in the position to approach the question of transendothelial transport and, as the atherogenic precursors become better defined, to begin applying these same techniques more directly to study of atherogensis.

Concluding Remarks

As indicated above, the work this year has added further evidence supporting the working hypothesis presented at the beginning of this report, namely, that arterial smooth muscle cell proliferation and the metabolism of certain low density lipoproteins occur in regions of increased concentration of certain atherogenic precursors arising from the blood, particularly in areas of increased transendothelial transport. Much of our effort over the ensuing years will be directed to a more detailed identification of these atherogenic precursors, to establishing the physical chemical laws governing their transport and accumulation at certain sites of the wall, and to designing studies to gain deeper insight into the cellular responses to these transmural concentration distributions.

These efforts have required, and will continue to require collaborative ties among the B/I/D (VRB (DRS), Clinical Pathology Dept. (CC), BEIB (DRS), DCRT, Molec. Dis. (H), SLAM (H), and Heart Surgery (H)), as well as extramural contractural support.

We have reviewed the economics of contract support for this program in an effort to seek alternative, less expensive approaches to our objectives. In view of the very real constraints on both positions and space that are likely to continue for the foreseeable future, we shall have a continuing need for our service contracts, particularly in the areas of electron microscopy, preparative lipoprotein chemistry and radio labelling of proteins. Thus, for the present at least, the service contracts appear to be the only, if not the most economical source for these essential program supports.

In the area of research contracts, which we use for much of our colony work, certain alternatives appear possible in the near future. We are eagerly seeking scientific collaboration with certain members of the Veterinary Resources Branch (DRS) in an effort to develop a more efficient and scientifically manageable solution to our projected need for experimental animal colonies. The soaring costs for these essential program support areas are of major concern to us and will be under continual review for more economical alternatives.

Project No. Z01 HL 02801 03 SEA
1. Office of Director on Intramural Research
2. Section on Experimental Atherosclerosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The arterial intimal tissue responses following endothelial injury.

Previous Serial Number: NHLI-199

Principal Investigator: Donald L. Fry, M.D.

Other Investigators: Victor J. Ferrans, M.D., Ph.D.

Cooperating Units: Section on Pathology, NHLI

Project Description

Objective: To study the functional and structural changes in the arterial wall following the erosion of the endothelial surface by increased fluid shear stress.

Methods Employed: Detailed methodology for this project appears in last year's report. Briefly, the endothelial surface of the descending thoracic aorta in a group of dogs was discretely removed using an inflated arterial balloon. The animals were allowed to recover and were examined at selected times following endothelial cell erosion using both light microscopic and electronmicroscopic techniques to examine the sequences of events leading to repair of the damaged arterial surface. Concomitant estimates of trans-endothelial macromolecular transport were carried out.

Major Findings: The sequence of repair were as follows: There was immediate generalized platelet adhesion to the eroded surface which was followed in a matter of days by a rapid proliferation of modified smooth muscle cells. Albumin transport into the arterial wall was increased from 10 to 15 fold both in the acutely eroded surface as well as the surface subsequently covered with smooth muscle cell proliferation. At a point in time corresponding roughly with the reappearance of a normal endothelial cell cover, both the proliferative response and the increased trans-intimal transport of albumin decreased.

Significance to Bio-medical Research and the Program of the Institute: The atherosclerotic processes are classically characterized by fibromuscular hyperplasia and lipid deposition. The sequence of events leading to this thickened intima are not understood. The topography of intimal proliferative processes in the diseased state frequently corresponds to areas that

may have been exposed to increased mechanical stress and/or injury. If the mechanisms of these naturally-occurring processes in this important disease are to be understood, it is essential that we understand in greater detail the factors inducing and controlling the reparative processes occurring at the vascular interface.

Proposed Course of the Project: A large amount of histologic and electron microscopic material has been generated from the above studies and is still in the process of analysis. The major problem in the interpretation of these data is the lack of precision with which the exact extent and area of the initial injury can be defined, particularly in the chronic preparations. It is not possible without this information to establish unequivocally the exact relationship between the reappearance of the normal endothelial surface, the "shutting-off" of the smooth muscle cell proliferative response, and the reappearance of normal macromolecular transport into the vascular interface. A variety of new techniques are being devised which will allow us to create well-defined, controlled, discrete regions of endothelial cell erosion thus allowing us to define this important set of feedback mechanisms which play a central role in the atherogenic process.

Keyword Descriptors

Endothelial erosion; albumin transport mechanics; Evans blue dye; endothelial and intimal repair; atherosclerosis.

Honors and Awards

1. Invited speaker, American Heart Association, Council for High Blood Pressure Research conference entitled, "Arteriosclerosis and Hypertension: Observations and Speculation," in Cleveland, Ohio, October 12, 1974.

2. Invited speaker at meeting on Fluid Dynamic Aspects of Arterial Diseases, Ohio State University, September 18, 1974.

3. Invited as discussion leader on Gordon Research Conference on Atherosclerosis, June 1975, entitled "Fluid mechanics, transport phenomenon and the arterial wall."

Publications

None this year.

Project No. Z01 HL 02802 03 SEA
1. Office of Director of Intramural Research
2. Section on Experimental Atherosclerosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The relationship of arterial intimal Evans blue dye accumulation to surface reflectance and light absorption.

Previous Serial Number: NHLI-200

Principal Investigator: Donald L. Fry, M.D.

Other Investigators: None

Cooperating Units: None

Project Description

Objective: To establish and validate the relationship between the concentration of Evans blue dye in the vascular interface and the absorption of red light.

Methods Employed: A special reflectance measuring system was designed and constructed, such that opened vessel surfaces could be viewed under uniform illumination through a lens system on a large glass screen. A small aperture in the center of this screen led to the sensing surface of a photomultiplier tube. Appropriate light filters could be inserted in this light path as needed. The opened vessel surfaces were held in a specially designed servo-motor-driven tray submerged in saline, both to act as a metabolic supporting milieu for the vessel surface, as well as providing a nonreflecting optical coupling to the photo-sensing system. Various regions of the vessel surface could then be maneuvered by the operator under visual control such that a very small region on the vessel surface could be placed under the aperture in the screen. With a Wratten 29 filter in the light path it was possible to measure the amount of red light that is absorbed and reflected from the vessel surface. By staining various regions on the vessel surface to varying depths it was possible to establish a monotonic relationship between the optical absorbance $\rho$ (negative log of the ratio of light intensity from stained surface to that from adjacent unstained surface) of the stained surface and time (duration of exposure to EBD-albumin complex). A reflectance model based on the Lambert-Beer law of light absorption was evaluated to represent this relationship by fitting model parameters to data obtained as dye was eluted back out of the surface using an untagged albumin solution. The final model that was chosen implicitly predicts the optical absorption coefficient for EBD from these fitted parameters and also the change in vessel surface area that occurs with stretch of the wall. The accuracies of these implications were tested by measureing the EBD absorption coefficient directly in an Aminco microphotometer and by measuring the correlation between measured changed in wall stretch with those predicted from reflectance measurements.



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Major Findings: It was found that the accumulation of EBD in the vascular intima bears an approximately logarithmic relationship to the amount of light absorbed as given by $M = k_1\rho + k_3\rho^3$, where M is the surface accumulation in n·moles/cm$^2$ and $\rho$ is the optical absorbance. The constants $k_1$ and $k_2$ were found to be 3.92 and 0.80, respectively.

The validity of the model was established by noting that the predicted value of the absorption coefficient (from the fitted parameters) was 0.128 as compared to 0.126 for the directly measured value. The linear regression of the predicted wall stretch data on directly measured values had a slope of 0.99, a negligible intercept, and a correlation coefficient of 0.99. We conclude that the model represents the actual physical state with good precision.

Significance to Bio-medical Research and the Program of the Institute: When Evans blue dye is injected intravenously into an animal virtually all of the dye becomes bound to serum albumin. Knowing the molar ratio of Evans blue dye to albumin permits the use of Evans blue dye as a measurable visual tag for albumin. Measurement of the amount of Evans blue dye in the intima thus can be used as a measure of transvascular macromolecular transport. We have shown that the atherosclerotic process is greatly enhanced in regions of increased transport. The ability to quantify the topography of permeability in the opened vascular interface with this relatively simple reflectometric scanning technique opens many new areas in the study of atherogenesis heretofore closed.

Proposed Course of the Project: This methodology will be applied first to studies designed to quantify the correlation between the topography of vascular permeability and the topography of the atherosclerotic lesions in animals. The second major area of application will be to quantify the permeability changes that occur with various altered hemodynamic events known also to be related to an increased atherosclerotic process. A manuscript is in preparation.

Keyword Descriptors

Evans blue dye; intimal Evans blue dye accumulation; optical absorption coefficient (Evans blue dye).

Honors and Awards

1. Invited speaker at meeting on Fluid Dynamic Aspects of Arterial Diseases, Ohio State University, September 18, 1974.

2. Invited speaker American Heart Association, Council for High Blood Pressure conference entitled, "Arteriosclerosis and Hypertension: Observations and Speculation," in Cleveland, Ohio, October 12, 1974.

3. Invited as discussion leader on Gordon Research Conference on Atherosclerosis, June 1975, entitled "Fluid mechanics, transport phenomenon and the arterial wall."

Publications

None this year.

Project No. Z01 HL 02803 02 SEA

1. Office of Director of Intramural Research
2. Section on Experimental Atherosclerosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The study of arterial transport processes in an in vitro life support system.

Previous Serial Number: NHLI-197

Principal Investigator: Donald L. Fry, M.D.

Other Investigator: Robert W. Mahley, M.D., Ph.D., and Robert J. Lutz, Ph.D.

Cooperating Unit: Biomedical Engineering and Instrumentation Branch, Division of Research Services

Project Description

Objective: This is a continuing effort, the objective of which is to develop an in vitro arterial support system for study of transvascular transport mechanics and short range metabolic mechanisms.

Methods Employed: A device has been developed which consists of a leaded glass chamber, the environment of which is thermostatically and humidistatically controlled by using appropriate electronic feedback systems. A carefully removed arterial segment is placed in a specially designed holding device which permits control of the mechanical constraints on the blood vessel as well as anchoring the vessel for application of a "well assembly" and "pressure manifolding system" in the above chamber to permit selected areas of the opened vascular interface to be studied under controlled, chemical, thermal and mechanical conditions.

Major Findings: Tissues studied in the above system have been found to remain viable by both physiological and ultrastructural criteria at least two hours. The tissue should be viable for periods in excess of 8 to 12 hours with provision for profusion of the adventitial surface of the vessel and refreshment of the milieu in contact with the endothelial surface. The macromolecular conductance of the vascular interface for albumin has been found to be greatly increased by subtle mechanical injury, temperatures in excess of 40°C, stretching the surface in excess of 200%, exposing the surface to saline, exposure to certain foreign proteins, brief exposure to air, and exposure to various simple polar solvents such as ethanol. Moderate increases in conductance were produced by concentrations of epinephrine, noradrenalin, and histamine in levels of approximately one order of magnitude above "physiologic" serum levels. Serotonin, adenosine diphosphate do not appear to alter the wall conductance.



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Significance to Bio-medical Research and the Program of the Institute: This is an in vitro technique that has been developed in which the trans-endothelial, intimal and medial transport processes of arterial tissue can be studied under controlled mechanical, thermal and chemical conditions for periods of at least two hours and probably considerably longer with continuous refreshment of the milieu and purging of the adventitial surface. This degree of experimental control is not possible in the in vivo situation and, therefore, this new methodology opens many new avenues for studying transport mechanics otherwise not available. Evidence described elsewhere from this laboratory strongly suggests that increased interfacial transport of macromolecules is one of the central factors in the atherogenic process. Consequently, this new methodology should lead to important new insights in this disease process.

Proposed Course of the Project: The above device will be further developed so that the studies may be carried out for extended periods of time to permit evaluation of transport processes of substances having very low transvascular flux such as lipoproteins. The studies described above in which a number of vasoactive substances have been screened for their activity will be continued. These, as well as other substances, such as angiotension, other catecholamines, divalent cations, fibrinolysin, various proteases, lipases, hyaluronidase, chondroitenase, neuraminidase and so forth, must be evaluated on a quantitative basis in view of their potential atherogenic activity. The exact methodology for adventitial purging is in the process of development and will be continued.

Keyword Descriptors

Atherosclerosis; transvascular transport mechanics; endothelial surface, endothelial permeability; organ support system.

Honors and Awards

1. Invited speaker at meeting on Fluid Dynamic Aspects of Arterial Disease, Ohio State University, September 18, 1974.

2. Invited speaker American Heart Association, Council for High Blood Pressure Research conference entitled, "Arteriosclerosis and Hypertension: Observations and Speculation," in Cleveland, Ohio, October 12, 1974.

3. Invited as discussion leader on Gordon Research Conference on Atherosclerosis, June 1975, entitled "Fluid mechanics, transport phenomenon and the arterial wall."

Publications

None this year.

Project No. Z01 HL 02804 01 SEA
1. Office of Director of Intramural Research
2. Section on Experimental Atherosclerosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The arterial ultrastructure in areas of increased and decreased transvascular transport.

Previous Serial Number: None

Principal Investigator: Donald L. Fry, M.D.

Other Investigators: None

Cooperating Unit: Tousimis Research Laboratory under contract #NIH 73-C-535-CC

Project Description

Objective: To establish the ultrastructural characteristics of the endothelial surface and subendothelial tissues in areas of increased and decreased transendothelial transport of Evans blue dye tagged albumin.

Methods Employed: The topographic distribution of increased transendothelial albumin flux was determined using the Evans blue tagging technique in four, two year old foxhounds and in two, one year old minipigs by administering the dye twenty-four hours prior to study. At the time of study each animal was anesthetized with pentobarbital, and both the axillary arteries and inferior vena cava were cannulated for pressure profusion with buffered saline and fixation with three percent buffered glutaraldehyde for one hour. The arterial trees were then carefully dissected free and sets of specimens were removed for transmission and scanning electron microscopy from areas of increased transendothelial albumin transport and from areas of normal transport.

Major Findings: By scanning electron microscopy, the tissues from normal transport areas showed a highly ordered endothelial surface pattern with distinct cell borders while tissues from high transport areas showed a high degree of disorder and pleomorphism with poor definition of cell borders. Transmission electron microscopy showed normal endothelial architecture in both types of regions but differences in the subendothelial space. Normal regions demonstrated a more ordered and densely packed pattern of intimal connective tissue (collagen, elastic fibers, and elastin) whereas tissues from high transport areas showed a much looser connective tissue pattern with many open spaces characteristic of moderate interstitial edema.

Significance to Bio-medical Research and the Program of the Institute: The localized development of the atherosclerotic process has been shown to correspond to regions of antecedent increased transendothelial transport of

albumin and perhaps of other macromolecules such as lipoproteins. The above observations suggest that this disease process may result from increased flux of atherogenic precursors across regions of structurally and functionally abnormal endothelial cells.

Proposed Course of the Project: These data are still in the process of analysis. Studies are in progress to define the nature of the endothelial surface changes and factors causing these changes.

Keywork Descriptors

Endothelial surface; atherosclerosis; endothelial ultrastructure; endothelial permeability.

Honors and Awards

1. Invited speaker at meeting on Fluid Dynamic Aspects of Arterial Diseases, Ohio State University, September 18, 1974.

2. Invited speaker American Heart Association, Council for High Blood Pressure Research conference entitled, "Arteriosclerosis and Hypertension: Observations and Speculation," in Cleveland, Ohio, October 12, 1974.

3. Invited as discussion leader on Gordon Research Conference on Atherosclerosis, June 1975, entitled "Fluid mechanics, transport phenomenon and the arterial wall."

Publications

None this year.

Project No. Z01 HL 02805 01 SEA

1. Office of Director of Intramural Research
2. Section on Experimental Atherosclerosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The mechanics of albumin transport into the arterial intimal medial space.

Previous Serial Number: None

Principal Investigator: Donald L. Fry, M.D.

Other Investigators: Robert W. Mahley, M.D., Ph.D., and Karl Weisgraber, Ph.D.

Cooperating Units: None

Project Description

Objective: To establish a valid physical chemical model for macromolecular transport into the arterial wall.

Methods Employed: A specially designed in vitro test system described elsewhere was used to measure the flux of 125 Iodine tagged albumin and Evans blue dye tagged albumin into the arterial wall. This system permitted the arterial surface to be exposed to the tagged albumin under controlled conditions for various selected periods of time. Special experimental maneuvers were devised such that it was possible to study the uptake of albumin, not only as a function of time, but also as a function of wall stretch and of transmural pressure.

Major Findings: The accumulation of tagged albumin in the arterial wall under conditions of zero transmural pressure was found to vary with the square root of time. This relationship was found not only for $I^{125}$ tagged albumin but also for the accumulation of Evans blue dye. In some studies uptake was measured using doubly tagged albumin so that the relationship between albumin uptake and Evans blue dye accumulation could be defined explicitly. It was found that Evans blue dye does not alter the uptake of $I^{125}$ albumin and that, although both follow a square root law, the accumulation of Evans blue dye for all time periods was approximately four to five times greater than for the $I^{125}$ tagged albumin. Moreover, studies of the rate with which Evans blue dye diffuses back out of the intimal surface showed that the rate of elution in albumin was over ten times that in a saline milieu indicating that albumin acts as the principal, if not sole, vehicle for transport of Evans blue dye in the intimal space as well as in the blood phase. These data indicate that Fick's Second Law of Diffusion in a two-phase system can be used to explain all of the above observations, provided that, in the case of the albumin-Evans blue dye complex, one incorporates a binding coefficient to represent the ratio of

bound Evans blue dye to diffusible (on albumin) Evans blue dye into the intimal space. Thus the model for albumin transport in the intimal space can be represented by $\partial c/\partial T = D/(\beta+1)\partial^2 c/\partial x^2$ where c is the concentration of the diffusing molecular species of interest, D is the coefficient of diffusion for albumin in the tissue space, $\beta$ is the aforementioned binding coefficient which for the Evans blue dye-albumin complex in the wall would be approximately 15 ($\beta$ would be zero for the $I^{125}$ albumin). This model represents the diffusive flux into the arterial wall that is driven by an electrochemical gradient.

In the arterial system, a second driving force in addition to the above exists, i.e., the pressure acting across the wall. This was studied by measuring the uptake of tagged albumin as a function of imposed transmural pressure differences both in positive and negative directions. Comparison of the uptake curve for the positive transmural pressure to that for the negative transmural pressure allowed calculation of the increment increase in flux into the wall related to the pressure alone. The pressure driven flux into the wall was found to be of the same order as that for the steady state diffusive flux into the wall.

Significance to Biomedical Research and the Program of the Institute: A large body of evidence both from this laboratory as well as from others indicate that the atherogenic precursors probably come from the blood phase into the wall as various complexes with proteins. There is also some evidence that dissociation in the intimal space may occur with preferential binding of lipid substances in the tissue matrix. While albumin itself can carry both fatty acids and some cholesterol, its major interest to us is related to its use 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 for the first time to place macromolecular transport in vascular tissue on firm physical-chemical grounds and allow us to sharpen our technical, physical, and mathematical tools which will be essential in subsequent studies of the transport processes for the atherogenic precursors (as these become better defined).

Proposed Course of the Project: The above studies will be expanded to include arteries other than the aorta, to explore the influence of vasoactive substances on the albumin transport process, and to establish more firmly the influence of other physical parameters such as temperature, pH, etc.

Keyword Descriptors

Transvascular macromolecular transport mechanics; atherosclerosis.

Honors and Awards

1. Invited speaker at meeting on Fluid Dynamic Aspects of Arterial Disease, Ohio State University, September 18, 1974.

2. Invited speaker American Heart Association, Council for High Blood Pressure Research conference entitled, "Arteriosclerosis and Hypertension: Observations and Speculation," in Cleveland, Ohio, October 12, 1974.

3. Invited as discussion leader on Gordon Research Conference on Atherosclerosis, June 1975, entitled "Fluid mechanics, transport phenomenon and the arterial wall."

Publications

None this year.

Project No. Z01 HL 02806 02 SEA

1. Office of Director of Intramural Research
2. Section on Experimental Atherosclerosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Blood velocity profiles and hemodynamic stresses in the aorta and its major branches.

Previous Serial Number: NHLI-208

Principal Investigator: Dali J. Patel, M.D., Ph.D.

Other Investigators: H. Bulent Atabek, Ph.D., and Sung C. Ling, Ph.D.

Cooperating Units: The Department of Aerospace and Atmosphere Sciences, The Catholic University of America

Project Description

Objective: 1) To measure, in vivo, blood flow fields in the left circumflex coronary artery (LCCA), and 2) to study the geometry, pressure area variations, and flow patterns at the aortic trifurcation area in dogs.

Methods Employed: 1) Pressure, pressure-gradient, pressure-radius relationships and arterial taper were measured on the LCCA of anesthetized and open-chested dogs under wide range of blood pressures and flow rates produced by intravenous infusion of Persantine. Velocity profile, wall shear and flow were computed from these data using a nonlinear theory of blood flow. Flow was also measured with an electromagnetic flow meter to provide an independent check.

2) To determine geometry and pressure-area relationship in the aortic trifurcation area, a 16 mm cine camera equipped with a high-power telephoto lens was employed. Motion pictures of the pulsating arteries were taken. These pictures also contained the image of a strip chart (reflected by a mirror into the view field of the camera) indicating the arterial pressure. At the end of filming the animal was sacrificed and the region of interest was filled with a casting material under the mean value of the in vivo pressure. The casts were taken out from the animal after hardening and sliced at regular intervals. Enlarged projections of these slides were used to determine variation of shape and cross-sectional area along the aorta, at the junction and along the branches. The developed films were mounted on glass slides. Diameters were measured (with a calibrated reticle inserted in the microscope eyepiece) and the corresponding pressures were determined from these films. Resulting information was used for determining the pressure-area relation.

Major Findings: Results for the LCCA indicated that (1) the flow profiles although less blunt than those in the descending thoracic aorta were still

nonparabolic; (2) wall shear in coronary arteries maintained a high value through diastole, and (3) during intravenous infusion of Persantine both coronary flow and wall shear increased. The peak values of the shear stress during these infusions could approach the yield stress values reported by Fry for the endothelial cells.

The study of the trifurcation area is still in progress. The geometry and the pressure-radius relationships of this region have been experimentally determined. The pressure-flow relationships are currently under study.

Significance to Bio-medical Research and the Program of the Institute: The role of hemodynamic stresses in the etiology of early atherosclerosis is well established by Fry. In order to study this role quantitatively we need to measure, in vivo, flow fields in the critical areas of the circulatory system. The present study of LCCA and aortic trifurcation area signifies a modest step towards this goal.

Proposed Course of the Project: The LCCA study is completed and will be submitted to Circulation Research for publication. The propagation of pressure and flow in the aortic trifurcation area will be studied.

Keyword Descriptors

Mechanical factors in atherosclerosis; local blood flow field; geometry of critical areas of circulatory system.

Honors and Awards

1. Visited Bulgaria as a member of the American delegation sent by the National Academy of Sciences to visit various institutions and discuss common research problems in Biomechanics with members of Bulgarian Academy of Sciences.

Publications

None

Project No. Z01 HL 02807 02 SEA

1. Office of Director of Intramural Research
2. Section on Experimental Atherosclerosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Vascular mechanics: arterial wall properties

Previous Serial Number: NHLI-207

Principal Investigator: Dali J. Patel, M.D., Ph.D.

Other Investigators: R. N. Vaishnav, Ph.D.

Cooperating Units: Department of Civil and Mechanical Engineering, The Catholic University of America, Washington, D. C.

Project Description

Objective: To study local and overall viscoelastic properties of the blood vessel wall under physiologic condition.

Methods Employed: Two different methods were developed to study two aspects of local rheology of the vascular intima and the endothelial surface. 1) The first method was designed to study the local compliance of the vascular intima. To this end, a segment of canine middle descending thoracic aorta (MDTA) was excised, slit-open longitudinally and stretched to in vivo dimensions in a stretch-rack. The vessel was supported on the advential side by a plaster-of-Parris slurry which was allowed to harden. Small forces (120 mg. typically) were then applied to various locations on the intimal surface using a modified analytical balance, one pan of which was replaced by a metal rod with a prodder tip with 0.19 mm diameter. The displacement due to indentation caused by the tip was measured as a function of time by means of a differential transformer. The indentation at the end of 30 seconds ($\delta_{30}$) was considered to be indicative of the local compliance of the intima and its substructure. A considerable number of segments were tested using this method at various sites on the intima including the regions adjacent to intercostal orifices.
2) The second method was designed to study the strength of the endothelium using saline jets. An instrument was designed to apply jets at specific sites on the endothelium by modifying a microscope with turret-mounted objectives, one of which was replaced by a jet nozzle (diam. 0.4 mm). A carefully handled aortic segment was stretched as above in a stretch rack and backed by a piece of lucite. Controlled jets of saline were then applied to the endothelium for various durations and the resulting lesions made visible by staining the tissue subsequently with Evans blue dye.

The overall anisotropic, nonlinear viscoelastic properties of tissue were studied using segments of canine MDTA and carotid artery and canine

jugular and human saphenous veins. In some instances the veins had been transplanted in the arterial circulation for varying periods prior to study. The method in this case consisted of applying various levels of intravascular pressure and longitudinal force to the segment and measuring photographically the resulting dimensions.

Major Findings: The findings on the local compliance of the vascular intima have been published (see publications). Specifically, the regions adjacent to the intercostal orifices were found to be less compliant than the regions far away from the orifices. This confirms the histological finding of the existence of collagen-rich intimal pads in the orifice regions. From the study of the lesions created by saline jets it was found that the endothelium could resist high normal stress but was susceptible to damage by relatively low shear stress. This was evident by the fact that an intermediate jet strength gave rise to an annular lesion consisting of an undamaged core where the jet hit directly and a ring of damaged endothelium where the shearing stress due to jet efflux exceeded the endothelial yield strength. Experiments are underway to refine the method and to quantify the endothelial yield strength using this method.

The work on the overall viscoelastic properties of artery yielded 10 arterial relaxation functions which fully describe the nonlinear viscoelastic properties of arteries under physiologic loading conditions. From the work on veins, it was found that the elastic behavior of veins is markedly different from that of artery, veins becoming highly nondistensible at arterial pressure but, nonetheless, being capable of resisting these pressures. A paper on elastic properties of venous segments has been submitted for publication to Circulation Research.

Significance to Biomedical Research and the Program of the Institute: Recent findings of Fry indicate that hemodynamic and local tissue factors probably play a significant role in the pathogenesis of atherosclerosis. Hemodynamic shear stresses, for example, can alter the permeability of the endothelium to the macromolecules in the blood. The jet studies should provide valuable information on the strength of the endothelial surface and its permeability to macromolecules. Likewise, it has been observed that regions of the vascular intima subjected to chronic high undirectional stresses appear to develop densely oriented collagen in the subendothelial region which appears to protect them from subsequent lipid deposition. The studies on local compliance of the vascular intima reported above are significant in that they provide a rheological counterpart to the histological finding stated above. The work on overall viscoelastic properties is the most general characterization of the mechanical properties of arteries available to date and should help in studying effect of aging, disease process and pharmacologic agents on tissue properties. The work on veins has considerable clinical significance in surgical procedures to replace diseased arterial segments.

Keywork Descriptors

Mechanical factors in atherosclerosis; endothelial surface strength; local blood vessel properties.

Honors and Awards

1. Participated in a symposium on "Recent Advances in Blood Rheology and Hemodynamics," held during the 26th International Congress on Physiological Sciences in New Delhi, India, 1974.

2. Participated in a satellite conference to the 26th International Congress of physiological sciences entitled "Circulatory and Metabolic Adaptations to Stress," in Bombay, India, 1974.

3. Invited to present a paper entitled "Nonlinear Viscoelastic Properties of Large Blood Vessels," International Conference on Cardiovascular System Dynamics, Valley Forge, Pa., April 8, 1975.

Publications

1. Janicki, J.S., Patel, D.J., Young, J.T., and Vaishnav, R.N.: Rheologic properties of blood vessels. In Research Animals in Medicine. DHEW Publication No. (NIH) 72-333, Wash., D.C., pgs. 573-582, 1973.(Publ.,1974)

2. Gow, B.S., Schonfeld, D., and Patel, D.J.: The dynamic elastic properties of the canine left circumflex coronary artery. J. Biomechanics, 7:389-395, 1974.

3. Patel, D.J. and Vaishnav, R.N.: Rheology of blood vessels. Proceedings of the International Union of Physiological Sciences, Vol. X, XXVI International Congress, New Delhi, 1974, pp. 82-83.

4. Gow, B.S. and Vaishnav, R.N.: A microindentation technique to measure rheological properties of the vascular intima. J. Appl. Physiol., Vol. 38, No. 2, February 1975, p. 344-350.

5. Plowman, F., Young, J.T., and Janicki, J.S.: An instrument for dynamic measurement of longitudinal stresses and strains in a blood vessel *in vivo*. Biorheology (in press).

Project No. Z01 HL 02808 02 SEA

1. Office of Director of Intramural Res.
2. Section on Experimental Atherosclerosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Trial of psychophysiologic techniques for the amelioration of hypertension.

Previous Serial Number: NHLI 206 (c)

Principal Investigators: Bernard L. Frankel, M.D.; Dali J. Patel,M.D.,Ph.D.; David Horwitz, M.D.

Other Investigators: William T. Friedewald, M.D.; Edward Freis, M.D.

Cooperating Units: Hypertension Endocrine Branch, NHLI; Clinical Trials Branch, NHLI; Veterans Administration Hospital.

Project Description

Objective: Studies reporting that blood pressure (BP) can be altered by psychophysiologic techniques such as biofeedback, autogenic exercises, and hypnosis have not provided definitive information on how long the effects were sustained at useful levels and usually were not adequately controlled. The present study is designed to determine whether a combination of psychophysiologic techniques can produce a sustained, therapeutically useful reduction in BP in patients with essential hypertension.

Methods: The study group will consist of 30 patients with uncomplicated essential hypertension who show mean diastolic BP levels of 90 to 105 while supine. Patients undergo an initial eight week period of evaluation during which BP is taken at least once a week by a nurse-observer blind to the patient's experimental status throughout the study. Thereafter, patients enter a sixteen-week study period during which weekly BP determinations continue. They are randomly allocated to one of three groups:

1) Active Treatment (AT) Group - In 20 laboratory sessions, AT patients are trained in the use of electromyographic and/or skin temperature biofeedback and diastolic BP feedback. They also learn autogenic exercises, relaxation training and auto-suggestion. During laboratory training sessions, pulse, BP, finger temperature, and frontalis muscle activity are monitored noninvasively. These patients also pursue a monitored daily home practice program to reinforce and generalize laboratory learning.

2) Pseudo-Treatment (PT) Group - Patients receive only pseudo-diastolic BP feedback arranged to convey a sense of success to the patient. They are seen as frequently as the AT patients. Physiologic monitoring is the same as for the AT patients but home practice is omitted.

3) No-Treatment Control (NTC) Group - NTC patients participate in a minimal program consisting of weekly BP determinations by the nurse-observer for 16 weeks.

Second Phase Studies: PT and NTC patients are offered the opportunity to participate in the AT protocol after completing their initial sixteen-week period, each patient serving as his own control. Patients in any of the three groups having at least a ten percent fall in mean diastolic BP are studied for an additional two months to determine if this decrease is sustained. Comparisons are based on the findings in the last six weeks of each period.

Associated Observations: Prospective observations of psychological and hemodynamic status are performed initially. Patients undergo a clinical interview, standard psychological tests, and determinations of cardiac output utilizing a $CO_2$-rebreathing technique. Findings will be correlated with clinical success in an attempt to delineate the characteristics predictive of success.

Major Findings: Studies of ten patients have been completed; two were in the active treatment group, three in the pseudo-treatment group, and five in the no-treatment control group. Of the two AT patients, one has shown a modest decrease in mean post-AT diastolic BP (from 93.7 to 90.5 mm Hg) whereas the other was a treatment failure. One of the three PT patients showed a modest decrease in mean post-PT diastolic BP (from 104.3 to 100 mm Hg); he, thereafter, participated in active treatment for sixteen more weeks and had a further comparable decrease (from 100 to 95.8 mm Hg). The two other PT patients showed no post-PT changes in diastolic BP; one continued with the AT protocol and showed an appreciable post-AT clinical response (from 94.7 to 86.7 mm Hg). There were no BP changes in the five NTC patients. The BP of one of these five then also remained unchanged after subsequent participation in the AT protocol. Ten additional patients are actively involved in the study.

Significance to Bio-medical Research and the Program of the Institute: These preliminary findings suggest that psychophysiologic techniques can reduce BP in some subjects. This is the first study designed with adequate controls to evaluate the specificity of such BP alterations and to establish whether they have the magnitude and persistence to make them clinically useful.

Keyword Descriptors

Hypertension, psychophysiology, biofeedback.

Honors and Awards: None

Publications: None

Project No. Z01 HL 02809 01 SEA

1. Office of Director on Intramural Research
2. Section on Experimental Atherosclerosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Quantitation of the apolipoproteins in plasma by two-dimensional immunoelectrophoresis.

Previous Serial Number: None

Principal Investigator: Robert W. Mahley, M.D., Ph.D.

Other Investigator: Thomas P. Bersot, M.D., Ph.D.

Cooperating Units: None

Project Description

Objective: To determine changes in the distribution of apolipoproteins in the plasma of control and cholesterol fed animals and man. Also to monitor changes in cholesterol induced hyperlipoproteinemia following hypolipidemic drug therapy.

Methods Employed: Monospecific antisera has been prepared to the "arginine-rich," A-1, and B-apoproteins of the rat lipoproteins and are in preparation for dog, miniature swine and human apolipoproteins. Laurell's two-dimensional quantitative immunoelectrophoretic procedures as revised by Versey and Davis has been modified for lipoprotein apoprotein quantitation. Our modification of the method, which includes delipidation of samples by triton, has overcome a major difficulty of apoprotein quantitation, i.e., failure to analyze hypertriglyceridemic plasma because of the presence of large, poorly migrating lipoproteins. In addition to allowing quantitation of all plasma samples, delipidation also reduced the possibility of masked antigenicity.

The rat plasma lipoproteins are the principal lipoproteins under investigation at this time. The plasma lipoproteins of rats are fractionated by ultracentrifugation into four major density classes - d<1.006 (VLDL); 1.006-1.019 (intermediate); 1.019-1.063 (LDL and $HDL_1$ or $HDL_c$) and 1.063-1.21 ($HDL_2$). The quantitative distribution of the various apoproteins is determined in each ultracentrifugal fraction for control chow fed animals vs. rats on various hypercholesterolemic diets. The hypercholesterolemic diets contain lard and cholesterol plus a bile acid (either cholic acid or taurocholate). In addition, some of the animals also receive propyl thiouracil (PTU). The alterations in lipoprotein metabolism following administration of hypolipidemic drugs is determined by analysis of changes in the apolipoprotein distribution.



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Major Finding: Using the two-dimensional quantitative electrophoretic procedure as modified, it has been determined that cholesterol feeding in association with taurocholate and PTU results in a five-fold elevation of the "arginine-rich" apoprotein in plasma. The increase in the "arginine-rich" apoprotein is associated with the occurrence of the beta-VLDL in the d<1.006 fraction. Administration of an experimental drug supplied by the Upjohn Company (U-41,792) results in a reduction of the "arginine-rich" apoprotein in the d<1.006 fraction and an increase in the $HDL_c$ lipoprotein.

Significance to Biomedical Research and the Program of the Institute: A relatively simple and rapid quantitative method for apolipoprotein analysis provides a useful tool to monitor changes in plasma lipoproteins induced by dietary manipulations and drug therapy. These studies will provide insight into changes in lipoprotein metabolism associated with the development of atherosclerosis following cholesterol feeding or the reversal of atherosclerosis following drug therapy.

Proposed Course: The project will continue along the lines indicated above. The methods will be extended to the analysis of human lipoprotein changes induced by diet and drug therapy.

Keyword Descriptors

Quantitative immunoelectrophoresis; apolipoproteins; dietary manipulation; drug therapy; experimental animals; hypolipidemic drugs.

Honors and Awards

None

Publications

None

Project No. Z01 HL 02810 04 SEA

1. Office of Director on Intramural Research
2. Section on Experimental Atherosclerosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Hyperlipoproteinemia and atherosclerosis: changes in plasma lipoproteins and apolipoproteins induced by cholesterol feeding in dogs, swine, rats, rabbits, and Patas monkeys.

Previous Serial Number: NHLI 201

Principal Investigator: Robert W. Mahley, M.D., Ph.D.

Other Investigators: Karl H. Weisgraber, Ph.D., and Donald L. Fry, M.D.

Cooperating Units: NHLI Contract #N01 HI-3-2926, Meloy Laboratories, Springfield, Virginia.

Project Description

Objectives: 1) To characterize the lipoproteins and apoproteins from control dogs, miniature swine, Patas monkeys, rats, and rabbits, and to compare these to changes induced by cholesterol feeding. 2) To correlate the type of hyperlipoproteinemia with the type, distribution and degree of atherosclerosis.

Methods Employed: The various animal species indicated above are fed diets which contain 0.5 to 2.0% cholesterol as described previously. Isolation of the plasma lipoproteins is accomplished by the combination of ultracentrifugation and Geon-Pevikon block electrophoresis. The purified lipoproteins were characterized with respect to electrophoretic mobility, immunochemical reactivity, size by electron microscopy, chemical composition and apoproteins. The apoproteins are isolated and purified by Sephadex and DEAE column chromatography. Analyses of the apoproteins include 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 have a similar lipoprotein response which is associated with the development of atherosclerosis. Animals on a low cholesterol diet serve as controls. The characteristics of the hyperlipoproteinemia associated with atherosclerosis are as follows: 1) The beta-VLDL become prominent lipoproteins. The B-VLDL are beta migrating lipoproteins in the d<1.006 fraction which resemble the beta-VLDL of human type III hyperlipoproteinemia particularly with respect to the prominence of the "arginine-rich" apoprotein. 2) LDL and the intermediate lipoproteins (IDL) are present in increased concentrations and are variably enriched in the "arginine-rich" apoprotein. These lipoproteins and the beta-VLDL may

represent remnants of intestinal lipoproteins induced to transport the dietary lipid. 3) A unique class of lipoproteins, which we have called $HDL_c$, are a consistent feature following cholesterol feeding. These lipoproteins are cholesterol-rich and contain the "arginine-rich" apoprotein and A-1. They lack the B-apoprotein. The $HDL_c$ are important regulators of sterol synthesis in aortic smooth muscle cells and fibroblasts (See project report entitled,"Tissue culture studies of aortic smooth muscle cells and skin fibroblasts: cell growth and metabolism in response to incubation with various lipoprotein classes."

Characterization of the "arginine-rich" apoprotein suggest homology of this protein among the species. They have a similar amino acid analysis and contain 12 moles% arginine. The "arginine-rich" apoprotein appears to play an essential role in cholesterol transport between lipoproteins and possibly between lipoprotein and the aortic wall (atherosclerotic lesion).

Significance to Bio-medical Research and the Program of the Institute: Characterization of cholesterol induced hyperlipoproteinemias 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.

Keyword Descriptors

Atherosclerosis; lipoproteins; cholesterol; diet; dogs; swine; rats; rabbits; monkeys.

Honors and Awards

Invited speaker at Lipid Metabolism Gordon Research Conference, entitled "Atherogenic and Non-Atherogenic Hyperlipoproteinemia Induced by Cholesterol Feeding in Dogs."

Invited lecturer at Bowman-Gray Medical Center, entitled "Cholesterol Induced Hyperlipoproteinemia and Atherosclerosis."

Publications

1. Mahley, R.W., and Weisgraber, K.H.: Canine lipoproteins and atherosclerosis. I. Isolation and characterization of plasma lipoproteins from control dogs. Circ. Res. 35: 713, 1974.

2. Mahley, R.W., Weisgraber, K.H., and Innerarity, T.: Canine lipoproteins and atherosclerosis. II. Characterization of the plasms lipoproteins associated with atherogenic and non-atherogenic hyperlipidemia. Circ. Res. 35: 722, 1974.

3. Mahley, R.W., Weisgraber, K.H., Innerarity, T., Brewer, H.B. and Assmann, G.: Swine lipoproteins and atherosclerosis. Changes in the plasma lipoproteins and apoproteins induced by cholesterol feeding. Biochemistry (In press).

Project No. Z01 HL 02811 04 SEA

1. Office of Director and Intramural Research
2. Section on Experimental Atherosclerosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Aortic metabolism of plasma lipoproteins.

Previous Serial Number: NHLI 202

Principal Investigator: Robert W. Mahley, M.D., Ph.D.

Other Investigators: Donald L. Fry, M.D., Karl H. Weisgraber, Ph.D.

Cooperating Units: None

Project Description

Objective: To determine 1) which classes of plasma lipoproteins are involved in aortic transport; 2) whether these lipoproteins are transported as intact macromolecules or hydrolyzed at the surface and components transported separately; 3) the fate of the lipoprotein components metabolized by the aorta.

Methods Employed: The in vitro transport method (described by Dr. D. L. Fry) is used to study aortic endothelial transport of the plasma lipoproteins under controlled conditions. Presently the dog is being used as the experimental model but we will soon extend this to the miniature swine as well. The components of canine plasma lipoproteins (VLDL, LDL, $HDL_1$ and $HDL_2$) are labelled 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 labelled. The $^{125}I$ lipid label is unstable. We are now able to label the protein moieties of the canine lipoproteins with $^{35}S$-methionine in vivo. At the same time we are able to label the phospholipids with $^{32}P$ orthophosphate. $^{14}C$-cholesteryl-esters and $^{3}H$-free cholesterol moieties of these lipoproteins are labelled in vitro by the exchange method of 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 (as described in a separate project report) 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 labelled plasma lipoproteins or a dried portion of the aorta following an in vitro transport study are placed in an oxygen combustion flask and ignited. $^{35}S$-methionine which is the protein tag and $^{32}P$ which is the phospholipid tag are converted to inorganic sulfate and phosphate, respectively. These isotopes remain in the flask and are quantitated together by standard double label liquid scintillation counting. The flask is heated to drive off the tritium in the form of $^{3}H_2O$ and $^{14}C$ in the form of $^{14}CO_2$. The $^{14}CO_2$ is collected by bubbling the gas through a base converting it to an insoluble carbonate and the $^{3}H_2O$ is collected on a condenser in an ice bath. Isotope recovery is greater than 90% and modifications are being made to increase the efficiency of the method.

Significance to Bio-medical Research and the Program of the Institute: It is agreed by most that 1) cholesterol within atheromata is derived largely from plasma lipoproteins and that 2) plasma lipoproteins can be detected within the same lesions. However, the mode of transport and the quantitative significance of lipoproteins in the lesions are far from clear. Serious questions remain as to whether the lipoproteins cross the endothelial surface intact or whether they are hydrolyzed at the surface with only some of the components entering the tissue. Our in vitro approach to this problem should shed light on this most difficult problem of atherosclerosis research as well as add to our knowledge of lipoprotein metabolism.

Proposed Course of the Project: The project will be continued along the lines indicated above. It will also be extended to the miniature swine and nonhuman primates for comparative studies.

Keyword Descriptors

Atherosclerosis; lipoproteins; endothelial transport; aorta transport; lipid metabolism.

Honors and Awards

None

Publications

None

Project No. Z01 HL 02812 06 SEA
1. Office of Director on Intramural Research
2. Section on Experimental Atherosclerosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Animal models for study of atherosclerosis.

Previous Serial Number: NIH 198

Principal Investigators: Robert W. Mahley, M.D., Ph.D.; Donald L. Fry, M.D.

Other Investigators: Victor J. Ferrans, M.D., Ph.D.; Joseph E. Pierce, DVM; Jere M. Phillips, DVM and David K. Johnson, DVM

Cooperating Units: Section on Pathology, NHLI; Section on Laboratory Animal Medicine and Surgery, NHLI; Veterinary Resources Branch, Division of Research Services; University of Missouri (Research Contract #NO1-HI-3-2947); and Colorado State University (Research Contract #NO1-HI-4-2903)

Project Description

Objective: To determine the suitability of a variety of animals as models for atherosclerosis as compared to the human disease.

Methods Employed: The animal models which have been studied in varying detail are the Patas monkey, miniature pig, dog, rabbit, and rat. The experimental conditions under which the pathologic processes in these animals can be made to resemble those in man have been detailed in previous project reports, including those referenced above, and therefore will not be described in detail here. Briefly, the disease can be induced in the rabbit, pig, and monkey, by feeding diets high in cholesterol and fat (lard); whereas disease can be induced in the dog and rat to a comparable extent only if hypothyroidism is also induced. In dogs the types of dietary fats are varied and included Wesson oil, pork lard, beef tallow, safflower oil and peanut oil.

Blood chemistries, which includes detailed lipoprotein studies, are monitored druing the experimental period. At termination each animal is examined in detail using the standardized necropsy procedures as described previously. Topographic distribution and histologic characteristics of aortic, coronary, and peripheral arteries are compared to human atherosclerosis. The comparative human material is derived from young adults dying tramatic deaths, unselected hospital cases, and patients with documented types of hyperlipoproteinemia.

Major Findings: Dietary induced atherosclerosis in our principal animal models - the dog, swine and monkey - have features which are strikingly similar to that in man. The topographic distribution of lesions in all species formed

a relatively characteristic and predictable pattern similar to that in man. Microscopic analysis showed early disease to be characterized by fatty streaking with only moderate intimal fibromuscular hyperplasia; whereas with longer duration of elevated serum lipids marked intimal fibromuscular hyperplasia occurred with the development of cell death, deposition of extracellular lipids and cholesterol crystals. Complicated disease appeared with the formation of atheromatous gruel under fibrotic caps, calcification and monocytic infiltration. The degree of disease in all the animals is directly correlated with the plasma cholesterol level and the appearance of a distinctive hyperlipoproteinemia. (Subject of progress report entitled, "Hyperlipoproteinemia and atherosclerosis. Changes in plasma lipoproteins and apolipoproteins induced by cholesterol feeding in dogs, swine, rats, rabbits, and Patas monkeys.")

The source of the dietary fat appears to be an important determinant of the distribution and morphologic characteristics of the disease in the dog. When beef tallow is substituted for pork lard as the principal dietary fat, the disease becomes much more severe and complicated in a shorter period of time with the development on many of the secondary complications as described in man. These complications include ulceration, thrombosis and embolism. Coronary artery disease with arterial occlusion and thrombosis is also accentuated when beef tallow is the source of dietary fat. The role of dietary fat, as a determinant of the type and severity of atherosclerosis, is being explored further in dogs and extended to swine, monkeys and rats.

Significance to Bio-medical Research and the Program of the Institute: The "atherosclerotic process" is, in fact, an ensemble of processes occurring at the cellular, physicochemical, biochemical, and biophysical level in the arterial intima. The purpose of this program is to identify as many of these fundamental processes as possible, establish which are relevant to those in man, and study these in great detail in the animal model, wherein the pertinent variables can be measured or controlled with a rigor not possible in man.

A clear definition of the role of diet in the development of atherosclerosis in man is of utmost importance.

Proposed Course of the Project: The pursuit of the above objectives will continue both at NIH in collaboration with the above mentioned cooperating units and with our contractor at Colorado State University.

Keyword Descriptors

Animal models; atherosclerosis; swine; dogs; rats; rabbits; monkeys; lipoproteins; cholesterol.

Honors and Awards

Invited speaker at the Deuel Conference on Lipid Metabolism, Carmel, Calif. entitled, "Suitability of Animal Models for Studies of Atherosclerosis," (Robert W. Mahley).

Publications: None

Project No. Z01 HL 02813 03 SEA

1. Office of Director of Intramural Research
2. Section on Experimental Atherosclerosis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Tissue culture studies of aortic smooth muscle cells and skin fibroblasts: cell growth and metabolism in response to incubation with various lipoprotein classes.

Previous Serial Number: NHLI-255

Principal Investigator: Thomas F. Bersot, M.D., Ph.D.

Other Investigators: Robert W. Mahley, M.D., Ph.D., Donald L. Fry, M.D.

Cooperating Units: None

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.

Methods Employed: 1) Standard techniques were used in determining cell proliferation in response to various classes of lipoproteins. 2) Electron microscopy was used to establish that cells were similar to smooth muscle cells as reported in the literature. 3) Cellular cholesterol production was assessed by measuring the activity of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, the rate limiting enzyme in cholesterol synthesis.

Major Findings: 1) Cultures of aortic smooth muscle cells from swine and dogs have been maintained up to ten generations without alteration of growth potential or morphologic characteristics. 2) When added to growth limiting medium in equal amounts with respect to cholesterol concentration lipoproteins from normo- and hyperlipidemic animals stimulated cell proliferation to the same extent. Very low density (VLDL) and low density (LDL) lipoproteins stimulated smooth muscle cell proliferation 7.8- and 6.1- fold respectively. High density lipoprotein (HDL) and a high density lipoprotein ($HDL_c$) induced by cholesterol feeding of swine stimulated cell proliferation 3.3- and 3- fold respectively. 3) In skin fibroblasts from normal human controls and swine aortic smooth muscle cells HMG CoA reductase activity was equally suppressed by human LDL and swine VLDL, LDL and $HDL_c$ when each lipoprotein class was added in equal amounts with respect to cholesterol concentration. HDL suppressed enzyme activity only when added in 50 fold greater concentrations with respect to cholesterol.



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Significance to Bio-medical Research and the Program of the Institute: In growth limiting medium lipoproteins containing the B-apoprotein are more effective in stimulating aortic smooth muscle cell proliferation. Animals and humans with high concentrations of these lipoprotein develop atherosclerosis. This predilection to develop atherosclerosis may be related to the ability of B-apoprotein containing lipoproteins to stimulate smooth muscle cell proliferation *in vivo*.

The ability of $HDL_c$ to suppress HMG CoA reductase is of interest because this lipoprotein contains no B-apoprotein. Previously it had been postulated that the B-apoprotein was essential and acted via a specific receptor on the cell surface of fibroblasts. The mechanism whereby lipoproteins suppress cellular HMG CoA reductase remains to be elucidated.

Proposed Course of the Project: Further studies with smooth muscle cells will be done to elucidate the mechanism of exogenous cholesterol metabolism.

Keyword Descriptors

Atherosclerosis; smooth muscle cells; cholesterol metabolism.

Honors and Awards

None

Publications

B. Greg Brown, Robert W. Mahley, and G. Assman: Swine Aortic Smooth Muscle in Tissue Culture: Cell Growth and Regulation of Cholesterol Synthesis in the Presence of Purified Swine Lipoproteins. Circulation Research (in press)

Annual Report of the Section of Pathology

Office of the Director

of the Division of Intramural Research

National Heart and Lung Institute

July 1, 1974 through June 30, 1975

This section is concerned primarily with structural alterations produced by various cardiovascular and pulmonary diseases. Structural alterations are studied at gross, light microscopic, ultrastructural and histochemical levels. Studies during this period focused on coronary, hypertensive, valvular and myocardial heart diseases.

## CORONARY HEART DISEASE

A continuing major undertaking of this laboratory is the examination of coronary arteries in a number of different conditions. Previous studies have focused primarily on the status of these arteries in patients with fatal acute myocardial infarction. During the past year or so the status of these arteries in patients with fatal pure angina pectoris and in individuals in whom the first coronary event was sudden death were also examined systematically. A major conclusion from these studies is that the myocardial response to similar degrees of coronary narrowing is quite different. For example, the degrees of coronary luminal narrowing are similar in patients with fatal angina pectoris, in patients with fatal acute myocardial infarction, and in patients in whom death is the first coronary event. A major mystery of coronary heart disease is to determine why the myocardium responds so differently to similar degrees of coronary luminal narrowing. Studies to attempt to answer this question are continuing in this laboratory.

In addition to studying the coronary arteries in patients with fatal ischemic heart disease, the coronary arteries are being examined systematically in patients of varying ages and sex who die from automobile accidents. Thus, it will be possible to have controls of coronary arteries for comparison with the patients with ischemic heart disease.

A previous study from this laboratory showed unequivocally that atherosclerosis is accelerated in patients on long-term corticosteroid therapy. There has been much discussion in the past couple of years regarding use of aspirin as a preventive of intraarterial thrombosis or specifically platelet aggregation. Presently, the coronary arteries in a number of patients who died of rheumatoid arthritis but who were treated for many years with high doses of aspirin are being examined to see if these individuals have less atherosclerosis than patients of similar age and sex not treated with aspirin or corticosteroid therapy.

In recent years, a number of reports have appeared describing patients with "myocardial infarction and angiographically normal coronary arteries." Patients reported with this combination were reviewed along with patients

studied in our laboratory at necropsy who had large myocardial scars but morphologically normal coronary arteries. A major implication of most of the previous reports was that acute myocardial infarction may occur in the presence of normal coronary arteries. This thesis was re-examined. Among 40 patients previously described with non-catheter induced myocardial infarction and angiographically normal coronary arteries, in none was coronary angiography performed at the time of myocardial infarction. Indeed, the interval between the onset of infarction and the performance of coronary angiography was longer than 1 month. In 5 additional patients, however, in whom coronary angiography was performed at the time of acute myocardial infarction, coronary angiography in each showed an obstructed coronary artery but repeat catheterization at later times disclosed angiographically normal coronary arteries. Thus, it was apparent that the status of the coronary arteries at the time of actual myocardial necrosis was uncertain in most reported patients. Indeed, a normal coronary arterial tree has never been demonstrated by angiography at the time of acute myocardial infarction. It was reasoned that there were at least 5 explanations to explain why a coronary tree might be entirely normal by angiography after healing of an acute myocardial infarction: 1) the acute myocardial infarction never occurred. Evidence is presented that this is quite unlikely. 2) Too large a myocardial mass or too little hemoglobin or too low a profusion pressure was present to supply the myocardium by a normal coronary tree. Although this explanation does explain the presence of myocardial scars in some patients with big hearts, the patients reported as having normal coronary arteriograms and acute myocardial infarction all had normal sized hearts, normal blood hematocrits and either normal or elevated blood pressures preceding the myocardial infarction. This explanation, therefore, is unlikely. 3) The coronary angiograms were misinterpreted. It is well known that angiography tends to underestimate the degree of coronary luminal narrowing but this explanation did not appear adequate for several reasons to explain the occurrence of myocardial infarction in most of the patients. 4) Coronary spasm caused the acute myocardial infarction. Patients with Prinzmetal's angina were reviewed and it was clearly shown that spasm has never been documented to cause acute myocardial infarction. 5) Acute myocardial infarction was caused by an occluding embolus which subsequently lysed or recanalized. Evidence was presented that this was the most likely explanation for the occurrence of "myocardial infarction and angiographically normal coronary arteries."

There have been many reports in the past describing histologic features of tuberous xanthomas but it has not been known whether or not the patients in whom the xanthomas were examined had normal- or hyper-lipoproteinemia. Thus, it was not known whether or not these xanthomas differed structurally in patients with type II versus let's say type III hyperlipoproteinemia. A tuberous xanthoma was examined histochemically and ultrastructurally from a patient with homozygous type II hyperlipoproteinemia. It was found that all the lipid was contained in foam cells. The cell types identified in the xanthoma were primitive mesenchymal cells, elongated perivascular and fibroblast-like cells, and macrophages which filled with lysosomes. The lipid was present in the xanthoma in 4 different forms, primarily in non-membrane-bound forms. It appeared that non-lysosomal lipid storage in foam cells is a characteristic tissue response to the underlying metabolic defect in type II hyperlipoproteinemia.

Much has been written from this Institute on Tangier's disease where it was initially described, but little structural information has appeared on this entity. Histologic, histochemical and electronmicroscopic studies were made of bone marrow, tonsil and jejunum from patients with Tangier disease. Four morphologically distinct types of lipid inclusions were observed in foam cells observed in these 3 types of tissues: 1) crystals of cholesteryl esters; 2) droplets composed of mixtures of cholesteryl esters and triglycerides; 3) ceroid, and 4) particles which corresponded in size to plasma chylomicrons and very low density lipoproteins (VLDL). Comparisons were made of the ultrastructure and histochemistry of foam cells in Tangier disease and in other lipid storage diseases. Plasma chylomicrons and VLDL were considered the important sources of lipid accumulated in foam cells in Tangier disease.

SYSTEMIC HYPERTENSION

During recent years in this section about 400 hearts are accessioned annually from patients with various fatal cardiovascular disorders. Most of the hearts are enlarged and by far the most common cause of the cardiomegaly has been hypertension. A number of conditions termed "the hypertensive diseases" were reviewed to see the frequency of a history of systemic hypertension and secondly to see the percent with cardiomegaly unexplained by any mechanism other than hypertension. The conditions reviewed were sudden coronary death, angina pectoris, acute myocardial infarction, cardiac complications particularly cardiac rupture of acute myocardial infarction, aneurysm of aorta, atherothrombotic obstruction of the abdominal aorta or of its branches, cerebrovascular accidents including atherothrombotic cerebral infarction, primary intracerebral hemorrhage, lacunar softenings, and Charcot-Bouchard aneurysms. It was clear from analyzing the numbers from patients with each of these various conditions that systemic hypertension is an even more common precursor of symptomatic vascular disease than previously realized from obtaining a history of hypertension or from recording one's blood pressure. In other words, the heart weight is a better indicator of hypertension than the history. It was clear that hypertension acts as a major risk factor to development of cardiovascular disease in two ways: 1) by increasing the deposition of atherosclerotic plaques in major arteries most commonly by causing luminal narrowing with resulting organ ischemia or infarction or both, and 2) by weakening the media of certain arteries causing aneurysms which may or may not rupture. Its effect on the arterial media is direct whereas its effect on the arterial intima is indirect. Hypertension is the only known major underlying factor in 2 conditions: intracerebral microaneurysm (with or without rupture) and dissecting aortic aneurysm (excluding patients with the Marfan and Marfan-like syndrome), both of which are associated with medial weakening or disruption. The more common consequence of hypertension, however, is its ability to increase the amount of atherosclerotic plaquing in various arteries. This effect, however, is indirect because population groups with normal (< 200 mg per 100 ml) serum cholesterol levels do not have more or larger atherosclerotic plaques than do normotensive persons in those populations.

VALVULAR HEART DISEASE

A continuing activity in this section has been study of patients with valvular heart disease to gain more information regarding the natural history of various conditions and secondly to gain information regarding prosthetic valves. Many reports are available regarding hemodynamic evaluation of various prosthetic valves but few have appeared evaluating these valves from a morphologic standpoint. Previous studies from this laboratory have focused primarily on the caged-ball prosthesis and on various tissue valves. During the past year a study of 61 patients with a poppet-disc valve was carried out. Detailed study of the early and late deaths of these patients clearly showed that the disc poppet prosthesis is not an ideal substitute cardiac valve. It clots, despite anticoagulant therapy, it is intrinsically stenotic, portions of it, i.e., the disc, degenerate and it causes hemolysis to erythrocytes. Currently, the porcine or pig valve is being studied and data will be accumulated on it during the following year.

An opportunity presented itself to study 2 patients who had had Hufnagel prostheses, the original "cardiac" valve inserted into the descending aorta. One of these patients died 11 and the other one 13 years after implantation of the Hufnagel prosthesis. Neither of these 2 patients nor any of the 3 previously reported long-term survivors with Hufnagel prostheses in descending aorta had prosthetic related complications. Because of the danger of excising the descending aortic prosthesis, it appears most reasonable not to remove the descending aortic prosthesis at any time in these patients if aortic valve replacement is subsequently performed.

Extensive studies in the past have been carried out in this laboratory on patients with valvular aortic stenosis. These previous studies showed that most individuals aged 15 to 65 with aortic stenosis had congenitally bicuspid valves and that 75% of them were male. During the past decade, 73 hearts have been collected with atretic aortic valves. This anomaly is the most common cause of death from congenital malformations during the first week of life. Among the 73 hearts, the patients ranged in age from 1 to 17 days (average 5) and 74% were boys. Among the 73 patients, 4 had normal or near normal sized left ventricles and 69 had hypoplastic ones. Among the 4 with normal or near normal sized left ventricles, all had ventricular septal defects and 3 had normally developed mitral valves. In contrast, among the 69 with hypoplastic left ventricles none had ventricular septal defects. A review of previous reports on aortic atresia failed to disclose any reports of ventricular septal defect or normal-sized left ventricle associated with aortic atresia and therefore a new classification of this condition was proposed.

In 1962 the specific cardiovascular lesion of carcinoid heart disease was described from this laboratory for the first time. Its description at that time was limited to its gross and histologic features. During the past year the endocardial lesion in carcinoid heart disease was studied by electron-microscopy. The cellular elements of the carcinoid plaque consist primarily of smooth muscle cells and the extracellular elements, of collagen fibrils, microfibrils, layers of fibrillar material and dense spicules. No elastic fibers or fibrin deposits are present. These observations suggest that carcinoid plaques, which occur exclusively in patients with the carcinoid

syndrome, result from stimulation of endocardial smooth muscle cells to produce collagen and basement membrane-like material.

The condition, rupture of a sinus of Valsalva aneurysm, is well known, but there is virtually no information on the occurrence of aneurysm of 1 of the 3 sinuses of Valsalva without rupture. An opportunity to study such a patient occurred during this past year. The patient, an 83-year-old man, had a large aneurysm involving 1 of the 3 sinuses of Valsalva. It was entirely an incidental necropsy finding. Thus, these aneurysms do not always rupture.

A common observation at necropsy in older hearts is papillary nodules on the valvular endocardium and occassionally on the mural endocardium. These lesions were examined in 3 patients by electronmicroscopy and a new term for this condition was presented, namely "endocardial papillary elastofibroma," to emphasize the features which are most conspicuous and which serve to differentiate this tumor from myxoma. These lesions had not been looked at ultrastructurally previously.

Diffuse endocardial fibroelastosis (EFE) is a rare congenital malformation of the heart, but focal EFE is common. Most patients with healed myocardial infarction have dense endocardial thickening over the area of myocardial scarring. The left ventricular endocardium from 10 patients with EFE was studied by electronmicroscopy. The elastic fibers were much larger in the 4 patients with congenital EFE than in the 6 patients with acquired EFE. The explanation for this difference, unfortunately, was not determined.

MYOCARDIAL HEART DISEASE

During the past several years a number of myocardial biopsies have been submitted to this unit primarily from D.C. General Hospital and primarily from patients with idiopathic cardiomyopathy. These biopsies have been evaluated from several angles but one not previously utilized was comparing the morphologic ultrastructural observations in the patients who were known to be habitual alcoholics to those patients known not to be habitual alcoholics. Examination of the 2 groups blindly showed no ultrastructural differences.

At cardiac operations performed at the Clinical Center biopsies of myocardium are taken routinely for electronmicroscopic and histologic studies. Some of these biopsies are derived from papillary muscle or left atrial appendage during mitral valve replacement and others are taken by direct biopsy from left ventricular free wall at the time of aortic valve replacement. In patients with hypertrophic cardiomyopathy a portion of left ventricular outflow myocardium is excised. As a consequence there is a large data base available of myocardial biopsies to study. Among 134 patients with cardiac hypertrophy of various causes the nuclear membranes of the cardiac muscle cells were studied and 3 distinctive abnormalities were observed: 1) increased foldings and convolutions; 2) nuclear pseudoinclusions formed by cytoplasmic organelles protruding into saccular invaginations of the nuclear membranes, and 3) intranuclear tubules. The increased foldings and convolutions of the nuclear membranes, and the nuclear pseudoinclusions appear to result from synthesis of nuclear membranes in excess of that needed to accomodate the

increase in nuclear volume which occurs in hypertrophy. Intranuclear tubules in cardiac muscle cells probably represent an extreme cellular response to the stimulus of hypertrophy.

In other myocardial biopsies intranuclear glycogen in myocardium was studied. Glycogen deposits within nuclei of hypertrophied and normal-sized cardiac muscle cells were observed in 6 (7%) of 90 patients with various cardiac diseases. The cells, however, containing intranuclear glycogen did not show damage or degeneration. It appears that under certain conditions the nuclei of cardiac muscle cells can acquire the capacity to synthesize glycogen but its exact function within the nucleus is as yet undetermined.

Bone marrow transplantation has been a recent intervention in the National Cancer Institute in treating patients with leukemia, aplastic anemia, immune deficiency disease or metastatic cancer. The hearts of 20 patients with these various disorders were studied both histologically and ultrastructurally. All had received bone marrow transplantation. The cardiac alterations were found to be similar to those which occur in patients with similar hematologic and neoplastic disorders who had not been treated with bone marrow transplantation.

PULMONARY PATHOLOGY

There has been considerable interest for a number of years in pulmonary disease by members of the pathology section of the National Heart and Lung Institute. Interest has focused primarily, however, on various pulmonary vascular changes produced by cardiac diseases. During the past year the clinical program of the Pulmonary Branch of the NHLI has gotten underway and, consequently, approximately 30 lung biopsies were submitted to the pathology section of the NHLI for examination. These biopsies were studied extensively histologically and samples of each were also studied by electronmicroscopy. In addition, some tracheobronchial washings were studied cytologically. These morphologic findings are correlated with the pulmonary function studies done during life on these patients. The opportunity to study extensively morphologically pulmonary parenchymal disease has added a new and very welcomed dimension to the NHLI pathology section. In addition, pulmonary parenchymal disease in patients from other institutes at NIH is also being studied much more thoroughly by the NHLI pathology section. These observations will form the nucleus of hopefully many future presentations.

Project No. Z01-HL-03001-01-OD-P
1. ODIR
2. Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Coronary Arteries in Ischemic Heart Disease

Previous Serial Number: None

Principal Investigator: William C. Roberts, M.D.

Other Investigators: None

Cooperating Units: None

Project Description: This article summarizes observations on coronary arteries in fatal ischemic heart disease and also summarizes observations on acute or recent lesions particularly in these patients: 1) among patients with fatal ischemic heart disease, thrombi are infrequent in patients dying suddenly and in those in whom the necrosis was limited to subendocardium; 2) thrombus is found in a coronary artery in about 60% of patients with fatal transmural acute myocardial infarction; 3) among patients with transmural myocardial necrosis, the major determinant of the presence of coronary thrombosis appears to be cardiogenic shock; 4) the larger the area of myocardial necrosis the greater the likelihood of coronary thrombosis; 5) when coronary thrombosis is associated with acute myocardial infarction, the thrombus is always located in the artery responsible for profusing the area of myocardial necrosis; 6) thrombi occur in fatal ischemic heart disease in coronary arteries which are already severely narrowed by old atherosclerotic plaques; 7) coronary thrombi in fatal acute myocardial infarction are usually occlusive, short, and located entirely in the major trunks.

Keyword Descriptors: Coronary thrombosis, coronary atherosclerosis.

Honors and Awards: None

Publication: Accepted for publication in Cardiovascular Clinics

Project No. Z01-HL-03002-01-OD-P
1. ODIR
2. Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Coronary Arteries in Fatal Coronary Events

Previous Serial Number: None

Principal Investigator: William C. Roberts, M.D.

Other Investigators: None

Cooperating Units: None

Project Description: This report emphasizes certain aspects of the coronary tree in patients with various types of fatal coronary events. Points emphasized are the role of coronary thrombosis in acute myocardial infarction. Two factors implicate coronary thrombosis as the precipitating cause of acute myocardial infarction: 1) the occurrence of coronary arterial thrombi in many patients with fatal acute myocardial infarction and 2) the location of the thrombus in the coronary arteries responsible for supplying the area of myocardial necrosis. Five factors, however, tend to indicate that coronary thrombosis is a consequence rather than the precipitating cause of acute myocardial infarction: 1) the low frequency of thrombi in patients dying suddenly with or without previous evidence of cardiac disease; 2) the increasing frequency of thrombi with increasing intervals between the onset of symptoms of acute myocardial infarction and death; 3) the absence of thrombi in fatal transmural acute myocardial infarction nearly as often as they are present; 4) the near absence of thrombi in fatal subendocardial infarction; 5) the occurrence of thrombi in high percentage only in patients with cardiogenic shock, most of whom have large transmural infarcts. It appears from study of 107 patients with fatal acute myocardial infarction in this laboratory that the key to coronary thrombosis just as the key to thrombosis occurring anywhere in the body is slow blood flow or relative stasis and sufficient time for the thrombus to form. The absence of these 2 factors may explain the absence of coronary thrombosis in the sudden coronary death cases and the increasing frequency of thrombosis as the interval from onset of symptoms of myocardial ischemia to death increases.

Keyword Descriptors: Coronary thrombosis, coronary atherosclerosis.

Honors and Awards: None

Publications: This report will appear as a chapter in a book entitled Cardiac Controversies to be published in 1975 by Springer-Verlag Publishers, New York.

Project No. Z01-HL-03003-01-OD-P
1. ODIR
2. Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Reports
July 1, 1974 through June 30, 1975

Project Title: The Coronary Arteries In Coronary Heart Disease

Previous Serial Number: None

Principal Investigator: William C. Roberts, M.D.

Other Investigators: None

Cooperating Units: None

Project Description: This report summarizes certain characteristic changes in the coronary arteries observed at necropsy in patients with fatal ischemic heart disease: 1) the coronary arteries are diffusely involved by atherosclerotic plaques; 2) with rare exception, at least 2 of the 3 major coronary arteries are narrowed >75% by old atherosclerotic plaque; 3) the atherosclerotic process is limited to the epicardial coronary arteries; 4) certain portions of the coronary tree tend to develop larger atherosclerotic plaques and, therefore, more narrowed lumens than other portions; 5) of the so-called 3 types of atherosclerotic plaques, namely lipid, fibrous, complicated, only the complicated plaque is responsible for causing significant (>75%) narrowing of the lumens of the coronary arteries; 6) the degree of coronary arterial luminal narrowing by atherosclerotic plaques and the extensiveness of the plaquing are similar in patients with fatal ischemic heart disease irrespective of the type of fatal coronary event; 7) the composition of coronary atherosclerotic plaques and the degree of coronary arterial luminal narrowing in patients with fatal ischemic heart disease appear similar irrespective of whether or not the blood lipoprotein pattern is normal or abnormal; 8) the shapes of lumens of atherosclerotic coronary arteries are quite variable; 9) and, although the number 1 risk factor to development of atherosclerosis in the western world, advanced age does not necessarily indicate the presence of severe coronary atherosclerosis.

Keyword Descriptors: Ischemic heart disease, atherosclerosis, hyperlipoproteinemia.

Honors and Awards: None

Publications: This piece is being published as a chapter in a book entitled Pathobiology Annals 1975 (Vol. 5) to be published in 1975 by Appleton-Century-Crofts.

Project No. Z01-HL-03004-01-OD-P
1. ODIR
2. Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Coronary Arteries in Ischemic Heart Disease

Previous Serial Number: None

Principal Investigator: William C. Roberts, M.D.

Other Investigators: Bernadine H. Bulkley, M.D.
Victor J. Ferrans, M.D., Ph.D.

Cooperating Units: None

Project Description: This report summarizes chronic lesions observed in the coronary arteries in patients with fatal ischemic heart disease and calls attention to acute lesions in coronary arteries other than thrombi. These latter lesions are 1) hemorrhage into an old atherosclerotic plaque. These were observed in 20% of nearly 200 patients studied in this laboratory with fatal ischemic heart disease. There was evidence in none of the patients, however, that hemorrhage caused any additional narrowing of the coronary lumen. 2) Coronary artery embolism. This was observed in 15 patients and criteria for the diagnosis of embolism was established. It was emphasized that clot nearly always is present in an intramural coronary artery when embolism is present and that in addition it occurs in the distal portions of the extramural coronary arteries. 3) Dissecting aneurysm (hematoma) of a coronary artery with and without associated dissection of aorta. Three patients were summarized in whom dissection was isolated to a coronary artery.

Keyword Descriptors: Dissecting aneurysm, coronary embolism.

Honors and Awards: None

Publications: This was published in La Revue de Medicine, Vol. 16:15-20, January, 1975. Complete title: Necropsy Observations on the Coronary Arteries in Ischemic Heart Disease.

Project No. Z01-HL-03005-01-OD-P
1. ODIR
2. Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Coronary Thrombosis in Myocardial Infarction

Previous Serial Number: None

Principal Investigator: A. Bleakley Chandler, M.D.

Other Investigators: Irving Chapman, M.D.
Leif R. Erhardt, M.D.
William C. Roberts, M.D.
Colin J. Schwartz, M.D.
D. Sinapius, M.D.
David M. Spain, M.D.
Sol Sherry, M.D.
Paul M. Ness, M.D.
Toby L. Simon, M.D.

Cooperating Units: Multiple ones

Project Description: In recent years the widely held concept that coronary thrombi cause myocardial infarcts has been seriously questioned. On the basis of pathologic studies, several reports have suggested that coronary thrombi do not cause infarcts but instead are the result of infarction. Should these findings become generally substantiated, the antithrombotic approach to the prevention and therapy of ischemic heart disease must be revised. This workshop was organized to examine more closely this issue and to sort out reasons for such divergent views of the role of thrombosis in the pathogenesis of myocardial infarction.

Keyword Descriptors: Coronary thrombosis.

Honors and Awards: None

Publications: American Journal of Cardiology 34:823-833, December 1974.

Project No. Z01-HL-03006-01-OD-P
1. ODIR
2. Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Thrombosis, Atherosclerosis and Ischemic Heart Disease

Previous Serial Number: None

Principal Investigator: William C. Roberts, M.D.

Other Investigators: Victor J. Ferrans, M.D., Ph. D.

Cooperating Units: None

Project Description: This report summarizes observations learned during the past couple of years in this laboratory suggesting that atherosclerotic plaques result, at least in part, from organization of thrombi: 1) the presence of known components of thrombi--namely fibrin and platelets--within atherosclerotic plaques; 2) the occurrence of known atherosclerotic plaques--namely foam cells, cholesterol clefts, pultaceous debris, calcium--in organized hematomas or known thrombi wherever they occur in the body; 3) the presence of multiluminal channels in vessels, a recognized consequence of organization of pulmonary thromboemboli--and presumably also coronary thrombi or emboli; 4) the major component of the complicated atherosclerotic plaque, i.e., that capable of causing significant luminal narrowing, in the coronary arteries of patients with fatal ischemic heart disease is fibrous tissue or collagen, not lipid and this is true whether or not hyperlipidemia is or was present; 5) experimentally induced thrombi under proper conditions may be transformed into atherosclerotic plaques closely resembling those observed in human coronary arteries. The above factors do not prove that thrombosis is the cause of atherosclerosis, but together they strongly suggest that organization of thrombi plays a major role in the development of the complicated atherosclerotic plaque.

Keyword Descriptors: Coronary thrombosis, coronary atherosclerosis.

Honors and Awards: None

Publications: Accepted for publication as a chapter in the book entitled Thrombosis, Platelets, Anticoagulation and Acetylsalicylic Acid to be published by Stratton Intercontinental Medical Book Corporation in 1975.

Project No. Z01-HL-03007-01-OD-P
1. ODIR
2. Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Acute Myocardial Infarction and Angiographically Normal Coronary Arteries

Previous Serial Number: None

Principal Investigator: Ernest N. Arnett, M.D.

Other Investigators: William C. Roberts, M.D.

Cooperating Units: None

Project Description: This report examines previous reports of myocardial infarction and angiographically normal coronary arteries and discusses possible explanations for the occurrence of "myocardial infarction and normal coronary arteriograms." There have been 45 reported patients with "myocardial infarction and angiographically normal coronary arteries:" in 5 the acute myocardial infarction was produced at the time of cardiac catheterization and in the other 40 patients acute myocardial infarction was unrelated to cardiac catheterization. In 39 of the 40 patients with non-catheter related infarction the interval between the onset of acute myocardial infarction and performance of coronary angiography was longer than 2 months. Thus, the status of the coronary arteries at the time when a portion of left ventricular myocardium was noted is uncertain. Indeed, a normal coronary arterial tree has never been demonstrated by angiography at the time of acute myocardial infarction. This report examines then how a coronary tree may be entirely normal by angiography after healing of an acute myocardial infarction. Five explanations are considered and evidence is presented that the acute myocardial infarction was caused by an occluding embolus which subsequently lysed or recanalized.

Keyword Descriptors: Coronary embolism, coronary spasm, coronary angiography.

Honors and Awards: None

Publications: Accepted for publication in Circulation.

Project No. Z01-HL-03008-01-OD-P
1. ODIR
2. Pathology Section
3. Bethesda,. Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Myocardial Embolus to Coronary Artery

Previous Serial Number: None

Principal Investigator: William J. Hammer, M.D.

Other Investigators: Victor J. Ferrans, M.D., Ph.D.
William C. Roberts, M.D.

Cooperating Units: Division of Cardiology, Department of Medicine,
Georgetown University, Washington, D.C.

Project Description: Although infrequent, emboli to coronary arteries usually consist of fibrin and platelets. Other dislodged material in coronary arteries have included calcific debris, clumps of neoplastic cells, suture and other foreign materials, and colonies of microorganisms. The usual consequence of coronary embolism is acute myocardial infarction. Of over 200 hearts examined systematically at necropsy in patients with fatal coronary heart disease, one was observed to have an embolus of necrotic myocardium in a coronary artery. The patient, a 73-year-old woman, died suddenly 10 hours after onset of symptoms (chest pain) of acute myocardial infarction. A precordial murmur was never audible. Necropsy disclosed rupture of one left ventricular papillary muscle and a clump of myocardium in the lumen of the right coronary artery. The embolized myocardium was similar to that observed in the ruptured papillary muscle.

Myocardial embolus, to our knowledge, has not been reported previously in a coronary artery. The coronary embolus in the present patient probably would have been missed had not the entire major coronary tree been examined histologically, a method used to study the coronary arteries at necropsy in all of our over 200 fatal cases of coronary heart disease.

Keyword Descriptors: None

Honors and Awards: None

Publications: Accepted for publication in Chest.

Project No. Z01-HL-03009-01-OD-P
1. ODIR
2. Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Tuberous Xanthoma in Type II Hyperlipoproteinemia

Previous Serial Number: None

Principal Investigator: Bernadine H. Bulkley, M.D.

Other Investigators: L. Maximilian Buja, M.D.
Victor J. Ferrans, M.D., Ph.D.
Gregory B. Bulkley, M.D.
William C. Roberts, M.D.

Cooperating Units: Surgery Branch, National Cancer Institute

Project Description: Histologic, histochemical and ultrastructural studies of a tuberous xanthoma from a patient with homozygous type II hyperlipoproteinemia showed that virtually all of the lipid was within histiocytic foam cells; no lipid was identified in interstitial regions or in blood vessels. Primitive mesenchymal cells, elongated perivascular and fibroblast-like cells, and lysosome-filled macrophages also were present within the xanthoma, indicating possible stages in the evolution of dermal mesenchymal cells into mature, cholesterol-rich foam cells. Morphologically the lipid was in 4 different forms: large droplets, which were the dominant form, and membrane-bound crystals, concentric lamellar bodies, and ceroid. The paucity of membrane-bound lipid forms, relative to the abundant free lipid droplets, indicated that lysosomal digestion was a minor metabolic pathway for the intracellular metabolism of lipid in the xanthoma. Thus, non-lysosomal lipid storage in foam cells is a characteristic tissue response to the underlying metabolic defect in type II hyperlipoproteinemia.

Keyword Descriptors: xanthoma, skin, type II hyperlipoproteinemia, pathology, ultrastructure

Honors and Awards: None

Publications: Bulkley, B.H., Buja, L.M., Ferrans, V.J., Bulkley, G.B., and Roberts, W.C.: Tuberous Xanthoma in Homozygous Type II Hyperlipoproteinemia. Archives of Path. (in press)

Project No. Z01-HL-03010-02-OD-P
1. ODIR
2. Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Pathology of Tangier Disease

Previous Serial Number: NHLI-243(c)

Principal Investigator: Victor J. Ferrans, M.D., Ph.D.

Other Investigators: Donald S. Fredrickson, M.D.

Cooperating Units: Molecular Disease Branch, National Heart and Lung Institute

Project Description: Histologic, histochemical and electron microscopic studies were made of bone marrow, tonsil and jejunum from patients with Tangier disease. The foam cells in these 3 types of tissues contained 4 morphologically distinct types of lipid inclusions: 1) crystals of cholesteryl esters; 2) droplets which were composed of mixtures of cholesteryl esters and triglycerides; 3) ceroid, and 4) particles which corresponded in size to plasma chylomicrons and very low density lipoproteins (VLDL). Detailed comparisons were made of the ultrastructure and histochemistry of foam cells in Tangier disease and in other lipid storage diseases. Examination of small, unmyelinated nerves in jejunal mucosa and submucosa revealed the presence of lipid deposits to the polyneuropathy which develops in patients with Tangier disease was discussed in detail. Plasma chylomicrons and VLDL are considered to be important sources of the lipid which accumulates in foam cells in Tangier disease.

Keyword Descriptors: Tangier disease, pathology, ultrastructure, bone marrow, intestine, skin, nerves, tonsils

Honors and Awards: None

Publications: Ferrans, V.J., and Fredrickson, D.S.: The Pathology of Tangier Disease. American J. of Path. 78: 101-158, 1975

Project No. Z01-HL-03011-01-OD-P
1. ODIR
2. Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Hypertensive Diseases. The Extent of Hypertension as a Risk Factor.

Previous Serial Number: None

Principal Investigator: William C. Roberts, M.D.

Other Investigators: None

Cooperating Units: None

Project Description: This report summarizes clinical frequencies of systemic hypertension and necropsy evidence of cardiomegaly in various cardiovascular conditions termed "the hypertensive diseases" because of their frequent association with systemic hypertension. Although long recognized as a major risk factor, systemic hypertension appears to be an even greater risk factor to development of various cardiovascular conditions than previously appreciated. Hypertension by itself appears to be the sole underlying factor in most cases of non-traumatic cerebral arterial or aortic rupture. In association with hyperlipidemia, hypertension clearly accelerates atherosclerosis and its devastating consequences.

This report demonstrates that systemic hypertension acts as a major risk factor to development of cardiovascular disease in 2 ways: 1) by increasing the deposition of atherosclerotic plaques (intimal lesions) in major arteries most commonly causing luminal narrowing with resulting organ ischemia or infarction or both, and 2) by weakening the media of certain arteries causing aneurysms which may or may not rupture. Its effect on the arterial media is direct whereas its effect on the arterial intima is indirect. Hypertension is the only known major underlying factor in 2 conditions: intracerebral microaneurysm (with or without rupture) and dissecting aortic aneurysm, both of which are associated with medial weakening or disruption. Reduction in blood pressure clearly leads to a reduction in the incidence of non-traumatic intracerebral hemorrhage and of dissecting aneurysm. The more common consequence of hypertension, however, is its ability to increase the amount of atherosclerotic plaquing in various arteries. This effect, however, is not direct because hypertension in population groups with normal serum cholesterol levels do not have more or larger atherosclerotic plaques than do the normotensive persons in those populations. Thus, hypertension accelerates or increases atherosclerosis only in hypercholesterolemic population groups and not in those with normal serum cholesterol levels.

Keyword Descriptors: Sudden coronary death, angina pectoris, acute myocardial infarction, aortic aneurysm, atherothrombotic obstruction of abdominal aorta or its branches, cerebrovascular accident, renal failure.

Honors and Awards: None

Publications: American Journal of Medicine (in press).

Project No. Z01-HL-03012-01-OD-P
1. ODIR
2. Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Cardiac Pathology after Valve Replacement Using Disc Prostheses

Previous Serial Number: None

Principal Investigator: William C. Roberts, M.D.

Other Investigators: Michael C. Fishbein, M.D.
Abner Golden, M.D.

Cooperating Units: Department of Pathology, Georgetown University, Washington, D.C.

Project Description: Clinical and necropsy observations are described in 61 patients in whom one or more cardiac valves had been replaced with discoid prostheses (Hufnagel type). The most common (31%) cause of death among the 45 patients dying early (<65 days from operation) appeared to be prosthetic disproportion, i.e., the prosthesis was too big for the aorta or ventricular cavity into which it was inserted so that inadequate space was present between the margins of the disc and the endocardium of ventricle or intima of aorta. Prosthetic thrombosis occurred in only 3 of the 45 patients dying early, but in each poppet movement appeared considerably altered. In contrast, thrombi were observed on a prosthesis in 14 of the 16 patients dying late (from 4 to 47 months [avg 21] postoperatively), but in none did the thrombi appear of sufficient size to alter poppet function. Excessive bleeding occurred in 11 of the 45 (24%) early deaths and was primarily related to the insertion of a patch in the root of aorta. Uncorrected valvular disease either by itself or by its ability to alter function of the prosthesis appeared responsible for death in 6 (13%) of the 45 patients dying early and in 2 (6%) of the 16 dying late. Insertion of a mitral poppet disc in a patient with uncorrected aortic regurgitation, even of mild degree, may be hazardous because the aortic regurgitant jet stream may interfere with proper function of the mitral disc. Likewise, insertion of a poppet disc only in the aortic valve position in a patient with combined aortic and mitral regurgitation may considerably increase the degree of mitral incompetence because the aortic prosthesis is intrinsically obstructive.

Disc wear or variance was observed in all but one prosthesis in place for >1 year. Although hemolytic anemia of significant degree was not observed in any of the 16 patients dying late, the occurrence of renal hemosiderosis in 13 of the 16 patients indicates that the poppet disc prosthesis is considerably traumatic to erythrocytes.



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Thus, the disc-poppet prosthesis is not an ideal substitute cardiac valve. It clots, despite anticoagulant therapy, it is intrinsically stenotic, portions of it, i.e., the disc, degenerate, and it causes hemolysis to erythrocytes.

Keyword Descriptors: None

Honors and Awards: None
Publications: American Journal of Cardiology, May 1975.

Project No. Z01-HL-03013-01-OD-P
1. ODIR
2. Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Observations After Insertion of Hufnagel Prostheses in Descending Aorta

Previous Serial Number: None

Principal Investigator: William C. Roberts, M.D.

Other Investigators: Michael C. Fishbein, M.D.

Cooperating Units: Department of Pathology, Georgetown University, Washington, D.C.

Project Description: Clinical and necropsy observations are described in 2 patients who died 11 and 13 years, respectively, after implantation of Hufnagel prostheses (the first ever used successfully in humans to treat cardiac valve disease) in the descending thoracic aorta. Despite the long implantation periods, there was no evidence of prosthetic degeneration or thrombosis or intravascular hemolysis. Although approximately 4000 Hufnagel descending aortic prostheses were distributed by the manufacturer for human use, data in only 55 patients in whom these prostheses were inserted were found in previous publications, and 26 of them had died. Of the 13 late deaths previously reported, no evidence of prosthetic degeneration or thrombosis was described in the 3 patients surviving >3 years (8, 10, 12.5 years, respectively). Since neither our 2 patients nor the other 3 reported long-term survivors had prosthetic-related complications and since the dangers of excising the descending aortic prosthesis are considerable, it appears most reasonable, as a rule, not to remove the descending aortic prosthesis at any time in these patients if aortic valve replacement is subsequently performed.

Keyword Descriptors: None

Honors and Awards: None

Publications: Chest (in press).

Project No. Z01-HL-03014-01-OD-P
1. ODIR
2. Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Aortic Valve Atresia: A Necropsy Study of 73 Cases.

Previous Serial Number: None

Principal Investigator: William C. Roberts, M.D.

Other Investigators: Lowell W. Perry, M.D.
Roma S. Chandra, M.D.
Stephen R. Shapiro, M.D.
Lewis P. Scott, M.D.

Cooperating Units: Division of Pediatric Cardiology, Department of Child Health and Development and Department of Pathology, Children's Hospital National Medical Center, Washington, D.C.

Project Description: Although a relatively uncommon lesion, aortic valve atresia is an important congenital cardiovascular malformation because it represents the least tolerated cardiac anomaly and, therefore, one extreme of the spectrum of congenital heart disease. Among infants with congenital malformations of the heart or great vessels during the first week of life, aortic valve atresia is the most common cause of death, the most common cause of congestive heart failure, and the cause of the largest hearts. This report describes the morphologic features of 73 hearts examined at necropsy with aortic valve atresia. The patients ranged in age from 1 to 17 days (average 5) and 74% were boys. Previous reports on aortic valve atresia described only the occurrence of a hypoplasitc left ventricle with or without atretic mitral valves. In the present study of 73 cases 4 were found to have normal or near-normal sized left ventricles rather than hypoplastic ones and 3 of them had normally developed mitral valves. All 4 patients with normal or near-normal sized left ventricles had ventricular septal defects whereas all the remaining 69 with hypoplastic left ventricles had intact ventricular septae. Because no previous descriptions have appeared of ventricular septal defect or normal-sized left ventricles associated with aortic atresia a new classification of this condition was proposed. In essence, there are 2 types of aortic valve atresia: in type I the left ventricle is hypoplastic and the ventricular septum is intact. Among our 69 patients with this type, 25 had atretic mitral valves and 44 had hypoplastic mitral valves. In type II, the left ventricular cavity is of normal size or near-normal size and one or more ventricular septal defects are present. Among our 4 patients with this type, 1 had an atretic mitral valve and 3 had a normally developed mitral valve. Although operative intervention in patients with hypoplastic left ventricles associated with aortic atresia is fruitless, it appears that operative intervention may be fruitful in the individuals with normal or near-normal sized left ventricular cavities.

Keyword Descriptors: Ventricular septal defect, mitral valve atresia.

Honors and Awards: None

Publications: Accepted for publication in the American Journal of Cardiology.

Project No. Z01-HL-03015-02-OD-P
1. ODIR
2. Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Endocardial Structure in Carcinoid Heart Disease

Previous Serial Number: NHLI-234(c)

Principal Investigator: Victor J. Ferrans, M.D., Ph.D.

Other Investigators: William C. Roberts, M.D.

Cooperating Units: None

Project Description: The carcinoid syndrome is associated with pathognomonic cardiac lesions which consist of plaque-like fibrous thickenings in mural and valvular endocardium. Ultrastructural study of these plaques in right atrium (2 patients) and tricuspid and pulmonic valve (1 patient) showed similar features. Most cellular elements were mature smooth muscle cells which varied from fusiform to stellate in shape and had greatly thickened, reduplicated basement membranes. Extracellular components consisted of: layers of normal-appearing collagen fibrils oriented parallel to the surfaces of the plaques and arranged in a cross-weaving pattern; 100 to 200 Å diameter microfibrils; layers of fibrillar material similar to basement membranes of smooth muscle cells, and dense spicules, 150 Å diameter and up to 800 Å in length. No elastic fibers or fibrin deposits were found. These findings suggest that carcinoid plaques result from stimulation of endocardial smooth muscle cells to produce collagen and basement membrane-like material; such stimulation may be intermittent, as evidenced by the layered arrangement of the plaques.

Keyword Descriptors: endocardium, pathology, ultrastructure, carcinoid syndrome

Honors and Awards: None

Publications: Ferrans, V.J., and Roberts, W. C.: Ultrastructure of Endocardial Plaques in Carcinoid Heart Disease. Human Pathology (in press)

Project No. Z01-HL-03016-01-OD-P
1. ODIR
2. Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Unruptured Sinus of Valsalva Aneurysm

Previous Serial Number: None

Principal Investigator: William C. Roberts, M.D.

Other Investigators: Michael C. Fishbein, M.D.
Robert T. Obma, M.D.

Cooperating Units: Skemp-Grandview Clinic, La Crosse, Wisconsin

Project Description: An unruptured congenital sinus of Valsalva aneurysm (behind the right aortic valve cusp) is described as an incidental necropsy finding in an 82-year-old man. Review of previous reports on aneurysms involving only 1 of 3 aortic sinuses disclosed that few cases have been described and that these lesions are rarely diagnosed during life. It is probable, however, that unruptured aortic sinus aneurysm (involving only 1 sinus) is more common than previous reports indicate, but that among patients with congenital sinus aneurysm, more likely than not, rupture will occur.

Keyword Descriptors: None

Honors and Awards: None

Publications: Accepted for publication in the American Journal of Cardiology to appear probably June 1975.

Project No. Z01-HL-03017-01-0D-P
1. ODIR
2. Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Endocardial Papillary Elastofibromas

Previous Serial Number: None

Principal Investigator: Michael C. Fishbein, M.D.

Other Investigators: Victor J. Ferrans, M.D., Ph.D.
William C. Roberts, M.D.

Cooperating Units: None

Project Description: The organization of cellular and extracellular components appeared similar and was distinctive in 3 endocardial papillary elastofibromas studied. Each tumor papilla contained: 1) a dense, central core of collagen and elastic tissue; 2) a peripheral, myxomatous layer with deposits of acid mucopolysaccharides, and 3) an overlying, hyperplastic layer of endothelial cells. Ultrastructural study of 1 tumor showed that the cells in all 3 zones had numerous cytoplasmic filaments, 100 Å in diameter, and dilated cisterns of endoplasmic reticulum; endothelial cells also had intercellular junctions and numerous pinocytotic vesicles. The myxomatous stroma varied from amorphous to fibrillar, and the collagenous cores showed focal degeneration. The name endocardial papillary elastofibroma is suggested to emphasize those features which are most conspicuous and which serve to differentiate this tumor from myxoma.

Keyword Descriptors: myocardium, valves, neoplasm, elastofibroma, pathology, ultrastructure

Honors and Awards: None

Publications: Fishbein, M.C., Ferrans, V.J., and Roberts, W.C.: Endocardial Papillary Elastofibromas. Archives of Path. (in press)

Project No. Z01-HL-03018-01-OD-P
1. ODIR
2. Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Ultrastructural Features of Endocardial Fibroelastosis

Previous Serial Number: None

Principal Investigator: Michael C. Fishbein, M.D.

Other Investigators: Victor J. Ferrans, M.D., Ph.D.
William C. Roberts, M.D.

Cooperating Units: None

Project Description: Histological and ultrastructural studies of left ventricular endocardium from 10 patients with endocardial fibroelastosis (EFE) revealed that the average size of elastic fibers in thickened endocardium was much larger in the 4 patients with congenital EFE than in the 6 patients with acquired EFE (secondary to ischemic heart disease in 2 patients, to prosthetic cardiac valves in 3, and to irradiation of the chest in 1). Both components of normal elastic tissue (central, amorphous cores and peripheral microfibrils) were present in endocardial elastic fibers of each patient. Ultrastructural identification of elastic fibers was greatly facilitated by staining with silver tetraphenylporphin sulfonate.

Keyword Descriptors: endocardium, fibroelastosis, pathology, ultrastructure

Honors and Awards: None

Publications: Fishbein, M.C., Ferrans, V.J., and Roberts, W.C.: Histologic and Ultrastructural Features of Primary and Secondary Endocardial Fibroelastosis. Submitted to Archives of Path. Laboratory Investigation (abstract) (in press)



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Project No. Z01-HL-03019-01-OD-P
1. ODIR
2. Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Morphologic Evaluation of Myocardial Protection

Previous Serial Number: None

Principal Investigator: Victor J. Ferrans, M.D., Ph.D.

Other Investigators: None

Cooperating Units: None

Project Description: The purpose of this project is to review histologic, histochemical and electron microscopic methods considered useful in the morphologic evaluation of procedures designed to prevent myocardial ischemic or metabolic injury during cardiac operations. Conclusions of this review are:

1. Transmural samples of myocardium should be studied, as the response of the ventricular walls to ischemic injury is not homogeneous.

2. Collection of samples should be continued until the injury reaches a stable end point.

3. Studies of myocardial protection should take into account the fact that ischemic injury is modified considerably by reflow phenomena.

4. Ultrastructural studies are indispensable, and histologic methods are of limited value in the morphologic evaluation of early myocardial injury.

Keyword Descriptors: myocardium, metabolism, ischemia, pathology, ultrastructure, histochemistry

Honors and Awards: None

Publications: Ferrans, V.J.: Morphologic Methods for Evaluation of Myocardial Protection. Annals of Thoracic Surgery, July, 1975



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Project No. Z01-HL-03020-02-OD-P
1. ODIR
2. Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Cardiac Morphologic Changes Produced by Ethanol

Previous Serial Number: NHLI-235(c)

Principal Investigator: Victor J. Ferrans, M.D., Ph.D.

Other Investigators: L. Maximilian Buja, M.D.
William C. Roberts, M.D.

Cooperating Units: None

Project Description: This study presents: 1) new ultrastructural observations (showing varying degrees of swelling of sarcoplasmic reticulum, mitochondrial damage, dilatation of T tubules, lipid accumulation, myofibrillar lysis and interstitial fibrosis) made on myocardial biopsies from patients with congestive cardiomyopathy and chronic alcoholism, and 2) a comprehensive review of all cardiac morphologic changes produced by the acute and chronic ingestion of large amounts of alcohol alone and alcohol plus cobalt-containing compounds (cobalt-beer cardiomyopathy) in humans and experimental animals.

Keyword Descriptors: myocardium, pathology, ultrastructure, cardiomyopathies, ethanol, drug effect

Honors and Awards: None

Publications: Ferrans, V.J., Buja, L.M., and Roberts, W.C.: Cardiac Morphologic Changes Produced by Ethanol. In Rothschild, M., Schreiber, S. and Oratz, M. (Eds.): Alcohol, Nutrition and Protein Synthesis. New York, Pergamon Press, 1975, pp. 139-185.



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Project No. Z01-HL-03021-01-3D-2
1. ODIR
2. Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Massive Myocardial Hemosiderosis

Previous Serial Number: None

Principal Investigator: Ernest N. Arnett, M.D.

Other Investigators: Arthur W. Nienhuis, M.D.
Walter L. Henry, M.D.
Victor J. Ferrans, M.D., Ph.D.
David R. Redwood, M.D.
William C. Roberts, M.D.

Cooperating Units: Molecular Hematology and Cardiology Branches,
National Heart & Lung Institute

Project Description: This report discusses a 23-year-old man with Blackfan-Diamond anemia. He received over 500 transfusions during his life and the effects of the massive deposition of iron in the myocardium is reviewed. This report emphasizes that heavy deposition of iron in myocardial fibers causes the myocardium to function abnormally and the present patient developed evidence of congestive failure and various arrhythmias. The distribution of iron in myocardium was described and the amount was quantitated biochemically and also studied ultrastructurally.

Keyword Descriptors: None

Honors and Awards: None

Publications: Accepted for publication in the American Heart Journal.

Project No. Z01-HL-03022-01-OD-P
1. ODIR
2. Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Cardiac Ultrastructure in the Cardiomyopathies

Previous Serial Number: None

Principal Investigator: Victor J. Ferrans, M.D., Ph.D.

Other Investigators: None

Cooperating Units: None

Project Description: This communication summarizes investigations on cardiac morphology in patients with hypertrophic cardiomyopathy (asymmetric septal hypertrophy, ASH) and in patients with congestive cardiomyopathies of various causes. In the ventricular septum of patients with ASH the muscle cells are severely disorganized and often run in different directions instead of in parallel. These cells are wider and shorter than in hypertrophy due to other causes and show increased cellular branching, extensive side-to-side intercellular junctions, widened Z bands, and evidence of formation of new sarcomeres. Some myofibrils are oriented obliquely or perpendicular to the longitudinal axis of the cell, and some myofilaments that originate from a single Z band of a given myofibril insert into Z bands of other myofibrils. These observations have led to the conclusions that: 1) ASH is a disease that involves the architectural arrangement of the muscle cell, particularly that of their contractile elements, and 2) this abnormal arrangement may lead to the generation of abnormal mechanical forces, which in turn may be responsible for the bizarre type of hypertrophy. In patients with obstructive ASH these abnormalities are either absent or present only to a very limited extent in muscle from the free walls of the left and right ventricles. In contrast to this, these changes were extensively distributed throughout both free walls in severely symptomatic patients with non-obstructive ASH. These observations suggest that the hypertrophy that develops in the ventricular free walls of patients with obstructive ASH is secondary to obstruction to outflow, and that cardiac functional limitation in these patients is due largely to this obstruction. The more diffuse abnormalities in patients with non-obstructive ASH suggest that these changes have a direct, important contribution to the cardiac functional impairment (failure of diastolic compliance) in these patients.

No distinctive or diagnostic lesion was demonstrated by histologic or ultrastructural study in myocardium of patients with congestive cardiomyopathies. These studies revealed degenerative changes, the severity of which generally correlated with the duration and degree of symptoms of

cardiac dysfunction. The ultrastructural changes in patients with congestive cardiomyopathies were found to occur to a variable extent in the late stages of ventricular hypertrophy due to other causes.

Keyword Descriptors: myocardium, cardiomyopathies, pathology, ultrastructure

Honors and Awards: None

Publications: Ferrans, V.J.: Cardiac Ultrastructure in the Cardiomyopathies. Part of the Symposium on the Cardiomyopathies in the Proceedings of the VII World Congress of Cardiology, Excerpta Medica, Amsterdam (in press)

Project No. Z01-HL-03023-01-OD-P
1. ODIR
2. Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Cardiac Structure in Hypertrophy

Previous Serial Number: None

Principal Investigator: Victor J. Ferrans, M.D.

Other Investigators: None

Cooperating Units: None

Project Description: This project consisted of a detailed review of cardiac structure in hypertrophy, with emphasis on ultrastructural alterations of myocardium in the three stages of hypertrophy (developing hypertrophy, stable hyperfunction and cellular exhaustion) and on quantitative data derived from stereological analysis of electron micrographs from animal models of hypertrophy.

Keyword Descriptors: myocardium, hypertrophy, ultrastructure

Honors and Awards: None

Publications: Ferrans, V.J.: Cardiac Structure in Hypertrophy. To be published as a book chapter in Cardiac Hypertrophy, Morkin, E. (Ed.), John Wiley & Sons



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Project No. Z01-HL-03024-01-OD-P
1. ODIR
2. Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Nuclear Membranes in Hypertrophied Human Myocardium

Previous Serial Number: None

Principal Investigator: Victor J. Ferrans, M.D., Ph.D.

Other Investigators: Michael Jones, M.D.
Barry J. Maron, M.D.
William C. Roberts, M.D.

Cooperating Units: Clinic of Surgery and the Cardiology Branch, National Heart and Lung Institute

Project Description: Nuclear membranes of cardiac muscle cells were studied in 134 patients with cardiac hypertrophy of various causes. Abnormalities observed consisted of: 1) increased foldings and convolutions; 2) nuclear pseudoinclusions formed by cytoplasmic organelles protruding into saccular invaginations of the nuclear membranes, and 3) intranuclear tubules. The increased foldings and convolutions of the nuclear membranes, and the nuclear pseudoinclusions, appear to result from synthesis of nuclear membranes in excess of that needed to accomodate the increase in nuclear volume which occurs in hypertrophy. Intranuclear tubules were found in 6 patients and consisted of tubular invaginations, 400 to 650 Å in diameter, of the inner nuclear membranes into the nucleoplasm. Some of these tubules were straight and cylindrical, and were associated with a peripheral layer of marginated chromatin; others were not associated with chromatin, appeared coiled and followed irregular courses. Intranuclear tubules in cardiac muscle cells probably represent an extreme cellular response to the stimulus of hypertrophy.

Keyword Descriptors: myocardium, hypertrophy, cardiomyopathies, nuclei, nuclear membranes, pathology, ultrastructure, nuclear tubules

Honors and Awards: None

Publications: Ferrans, V.J., Jones, M., Maron, B.J. and Roberts, W.C.: The Nuclear Membranes in Hypertrophied Human Cardiac Muscle Cells. Am. J. Pathol. 78: 427-460, 1975

Project No. Z01-HL-03025-02-OD-P
1. ODIR
2. Pathology Section
3. Bethesda, Maryland.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Intranuclear Glycogen in Myocardium

Previous Serial Number: NHLI-237(c)

Principal Investigator: Victor J. Ferrans, M.D., Ph.D.

Other Investigators: Barry J. Maron, M.D.
L. Maximilian Buja, M.D.
Nayab Ali, M.D.
William C. Roberts, M.D.

Cooperating Units: Cardiology Branch, National Heart and Lung Institute
Cardiac Laboratory, District of Columbia General Hospital

Project Description: Ultrastructural and cytochemical studies of myocardial biopsies disclosed the presence of glycogen deposits within nuclei of hypertrophied and normal-sized cardiac muscle cells in 6 (7%) of 90 patients with various cardiac diseases. Intranuclear glycogen appeared as β-particles, 160 to 360 Å in diameter, which either formed small aggregates or were dispersed in the nucleoplasm. Cells containing intranuclear glycogen did not show damage or degeneration. Glycogen particles in the cytoplasm of these cells were of the same size and appearance as those in the nuclei. Criteria for the ultrastructural identification of intranuclear glycogen are proposed, and it concluded that under certain conditions the nuclei of cardiac muscle cells can acquire the capacity to synthesize glycogen.

Keyword Descriptors: myocardium, pathology, ultrastructure, hypertrophy, glycogen

Honors and Awards: None

Publications: Ferrans, V.J., Maron, B. J., Buja, L. M., Ali, N., and Roberts, W. C.: Intranuclear Glycogen Deposits in Human Cardiac Muscle Cells: Ultrastructure and Cytochemistry. Journal of Molecular and Cellular Cardiology (in press)

Project No. Z01-HL-03026-02-OD-P
1. ODIR
2. Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Cardiac Lesions in Bone Marrow Transplantation

Previous Serial Number: NHLI-231(c)

Principal Investigator: L. Maximilian Buja, M.D.

Other Investigators: Victor J. Ferrans, M.D., Ph.D.
Robert G. Graw, M.D.

Cooperating Units: Experimental Hematology Section, Pediatric Oncology Branch, National Cancer Institute

Project Description: Cardiac pathologic findings were analyzed in 20 necropsied patients from a series of 26 patients with leukemia, aplastic anemia, immune deficiency disease or metastatic cancer who had been treated with bone marrow transplantation. Most cardiac alterations were similar to those which occur in patients with hematologic and neoplastic diseases who have not been treated with bone marrow transplantation, and consisted of: hemorrhage (12 patients), foci of necrosis associated with sepsis (9 patients), myocardial abscesses (4 patients), infective endocarditis (1 patient) and hemosiderosis (1 patient). Other cardiac alterations were more specifically related to bone marrow transplantation. Six patients exhibited a distinctive interstitial reactive change characterized by the presence of a pleomorphic population of lymphoid, histiocytic and Anitschkow cells. This alteration may have been induced by abnormal immune mechanisms, as suggested by the observation that 5 of the 6 patients with interstitial change had clinical evidence of graft-versus-host disease. Two patients developed fatal cardiac failure in the post-transplant period, and exhibited myocardial damage with histologic and ultrastructural features indicative of severe acute injury. Findings in these 2 patients consisted of: 1) necrotic muscle cells which exhibited multiple contraction bands, diastase-resistant PAS staining and intracellular fibrin deposits; 2) microthrombi which were composed of fibrin and, occasionally, of fibrin and platelets, and 3) extravasated erythrocytes and fibrin strands in interstitium. Clinico-pathologic analysis strongly suggested that the fatal cardiotoxicity in both patients resulted primarily from effects of high doses of cyclophosphamide (180 mg/kg and 270 mg/kg) which were administered as part of a newly developed regimen of combination chemotherapy-immunosuppression (B.A.C.T.). Our findings emphasize the need for more effective and less toxic antineoplastic and immunosuppressive therapy for patients who require bone marrow transplantation.

Keyword Descriptors: transplantation, bone marrow, myocardium, pathology, ultrastructure, drug effects

Honors and Awards: None

Publications: Buja, L.M., Ferrans, V.J., and Graw, R. G.: Cardiac Pathologic Findings in Patients Treated with Bone Marrow Transplantation. Human Pathology (in press)

Project No. Z01-HL-03027-01-OD-P
1. ODIR
2. Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Structural Basis of Abnormal Cardiac Function

Previous Serial Number: None

Principal Investigator: William C. Roberts, M.D.

Other Investigators: None

Cooperating Units: None

Project Description: This report, in essence, is a review of morphologic observations made in this laboratory during the past 10 years regarding coronary, hypertensive, valvular, idiopathic myocardial and pericardial heart diseases. The status of the major extramural coronary arteries in patients with fatal ischemic heart disease is summarized. Specifically, for fatal or even symptomatic ischemic heart disease to occur, at least 2 of the 3 major coronary arteries must be >75% narrowed by old atherosclerotic plaques. The myocardial response to severe coronary narrowing is highly variable and indeed no differences in degrees of luminal narrowing were observed among fatal cases of angina pectoris, sudden coronary death, and acute myocardial infarction. The frequency of histories of systemic hypertension in many cardiovascular conditions was reviewed and secondly the percent of patients with various cardiovascular conditions who have cardiomegaly were reviewed. It was emphasized that systemic hypertension is a far greater risk factor than previously emphasized by history of hypertension or measurement of elevated blood pressure if left ventricular hypertrophy is used as an indicator of hypertension rather than history or actual measurement of blood pressure. The morphologic features of various valvular lesions were reviewed and emphasis was placed on the fact that rheumatic heart disease is a less common cause of valvular heart disease than are non-rheumatic etiologies. Stress was placed on classifying valvular heart disease into the purely regurgitant lesions and into those with elements of stenosis because the etiology of the former are only 3 in number whereas the etiology of the latter were numerous. Morphologic features of the idiopathic cardiomyopathies, i.e., both the ventricular dilated and the non-ventricular dilated type (ASH), were reviewed. Likewise, various morphologic features of pericardial heart disease were reviewed.

Keyword Descriptors: Coronary heart disease, hypertension, valvular heart disease, myocardial heart disease, pericardial heart disease.

Honors and Awards: None

Publication: Chapter in a book entitled Clinical Cardiovascular Physiology edited by Herbert Levine to be published in 1975 by Grune and Stratton.

ANNUAL REPORT OF THE
SECTION ON THEORETICAL BIOPHYSICS
OFFICE OF THE DIRECTOR OF INTRAMURAL RESEARCH
NATIONAL HEART AND LUNG INSTITUTE
July 1, 1974 through June 30,1975

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.

Mathematical theory of renal function:

Since this report marks the fifteenth anniversary of our work on the theoretical analysis of renal function, a brief retrospective seems in order. From 1960 to 1970 we were primarily concerned with the theoretical analysis of solute cycling models of the renal counterflow system. In these models, originally proposed by Wirz and theoretically analyzed by Kuhn and Ramel, concentration of urine occurs by salt being actively transported out of ascending limb of Henle into the interstitium, whence it enters descending Henle's limb to be recycled. In this early work we analysed both the steady state and transient behavior of solute cycling systems and established that in single solute systems active salt transport out of ascending Henle's limb was necessary for concentration.

A large body of data, both from micropuncture and isolated tubule experiments, suggests that concentration of fluid in descending Henle's limb occurs primarily by water extraction rather than solute cycling. In this process solute from ascending Henle's limb and collecting duct enters the interstitium raising its osmotic pressure and so withdrawing water from descending Henle's limb and collecting duct. The water and salt supplied by the tubules are taken up by the blood vessels of the medulla and returned to the systemic circulation. Until 1970, attempts to model the water extracting process were singularly unsuccessful. The basic difficulty was the failure of solutions of equations describing such models to give simultaneous solute and water balance -- one or the other accumulating in the medullary interstitium.

In 1970 we discovered both the cure and the cause -- in that order. In earlier models medullary capillaries had been assumed to play a subsidiary role in the concentrating mechanism, conserving solute supplied by the renal tubules by a parallel vascular counterflow system that allows equilibration of solute in ascending and descending flows. In solute cycling models the classic view is essentially correct, but in models that concentrate by water extraction the capillaries are an integral part of the counterflow system. The difference between ascending and descending vascular volume flows must equal water taken up from the renal tubules and the difference between ascending and descending axial solute flows must equal solute taken up from the tubules. If one assumes that concentrations in ascending and descending

flows are identical one is led to a central core model of the medullary counterflow system in which blood vessels and interstitium are merged into a single tube, closed at the papillary end and open at the cortical where it is assumed to empty into the systemic circulation. Three parallel flow tubes, which can exchange with the core and with each other correspond to ascending limbs of Henle's loop, descending limbs of Henle's loops and collecting ducts. With this model we made the first coherent analysis of salt, water and urea movement and of free energy balance in the medullary counterflow system.

More or less simultaneously with the development of the central core model we discovered that the equations for the earlier models were inconsistant under the usual assumptions that were introduced to solve them. There are various ways to relieve this inconsistency. One is to permit volume flow in the interstitium parallel to the other flow tubes. With large hydraulic and solute permeabilities of the vasa recta this leads to the central core model. Alternatively, one can include hydrostatic and colloid osmotic pressure (neglected in earlier models) as a driving force for the transmural movement of solutes and water in medulla. This alternative was not feasible in 1970, because numerical methods for solving the equations describing such a complicated flow system were not available.

During the years 1970-71 and 1971-72 the analytical theory of the central core model was intensively developed and essentially completed. Among the principal results of this study were: 1) an analysis of the behavior of the medullary counterflow system for solute cycling, water extracting, and mixed modes of operation, 2) a study of concentration profiles and energetic requirements for different pump kinetics in ascending Henle's limb, 3) a new understanding of the role of urea in the concentrating mechanism.

Early in 1972 a detailed five year research plan was developed with the two closely related goals of expanding and deepening the general theoretical analysis of renal function and of developing a realistic computer simulation of renal function, which would relate membrane transport properties of the nephron to macroscopic function of the kidney. This program required a substantially increased committment of NHLI resources, which was justified on the basis of the following goals.

1) Increased understanding of renal function.

2) Improved diagnostic methods.

3) Better treatment of renal and cardiac disease.

4) Improvement in the design of artificial kidneys.

In carrying out the program the primary need was to develop numerical methods for solving the equations describing kidney models. To do this required expertise in numerical analysis and computer programming not possessed by NHLI at the outset. A cooperative program was initiated with

a DCRT mathematician who subsequently transferred to NHLI. We also have utilized cooperative and collaborative programs with mathematicians possessing required expertise at the University of Maryland, Louisiana Polytechnic Institute, and SUNY, Stony Brook, N. Y. This program has progressed extremely well and we have now developed several very efficient algorithms for solving difference equations describing water and solute transport in flow systems. These methods have been applied to a variety of models of the medullary counterflow system and have now been applied to a model of the whole kidney. With this model it has been possible for the first time to simulate behavior of the whole kidney as a function of hydrostatic pressure in renal artery, vein, and pelvis, protein concentration in arterial blood, and phenomenological equations describing transport of salt and water across nephron and capillary walls. The extension of our numerical method to models that consider several solutes and the distribution of nephrons into cortical and juxta medullary seems straight forward and with continued support we expect to meet our goal of developing a realistic computer simulation of the kidney by 1977.

Although our present models of the kidney and its various subsystems are at an intermediate level of sophistication, their study has already transformed our concepts of renal function. In the classic view, the functional unit of the kidney is the individual nephron; the one million or so nephrons making up the kidney act strictly in parallel to function as one giant nephron. Until the idea of countercurrent multiplication was introduced, it was thought urine formation could be understood by the sequential series processing of glomular filtrate by the various tubular segments. Folding the nephron in the countercurrent models introduced the concept that the more distal parts of the nephron can interact with the more proximal. In the solute cycling models this interaction is restricted to direct pairwise interaction of contiguous segments. This is reflected in the linearity of the equations describing these models and the sparseness of their connectivity matrix. In the central core models a new level of interaction is introduced. Functionally the counterflowing solution in the core links solute and water transport in noncontiguous segments of the nephron. Thus, solute transport out of ascending limb and collecting duct in the inner medulla induces water movement out of descending limb in the outer medulla; urea transport out of collecting duct is coupled to salt transport out of ascending Henle's limb; and active transport in cortex and outer medulla is coupled to passive transport in the inner medulla.

The numerical analysis of the detailed models, with tubules and capillaries exchanging either directly or via the interstitial space, have confirmed the insight gained from the central core model. It has also suggested the probable importance of the coupling of different types of nephrons via the vascular interstitial space. In addition, study of these detailed models has shown the importance of the transport properties of the capillaries.

The general thesis we have drawn from these studies is that the functional unit of the kidney is not the individual nephron but a nephrovascular unit consisting of a group of nephrons and their tightly coupled vasculature. The operation of this unit depends both on the segmental transport characteristics of the individual nephron and capillaries and the way in which these are coupled by the architecture of the unit. Our mathe-

matical techniques have reached the point where for the first time a synthesis of transport characteristics and coupling is possible.

A summary of specific results over the past twelve months follows:

Improvement of numerical methods for solving the difference equations describing various models continued as a major project. During the year a variety of partitioning schemes have been developed for solving models piecewise. A generalized sparse matrix routine was adopted for Newton-Raphson solutions. In addition several special sparse matrix inversion methods were developed. The efficiency of these schemes was compared for parallel flow tubes exchanging via an interstial space. Piecewise solutions were found to be accurate and most efficient in utilization of computer storage and time.

The improved numerical methods were used to obtain solutions for a model of the whole kidney and various models of the medullary counterflow system as described above.

Earlier work on solution of the time dependent equations by quasi-linearization was extended and compared with Newton type methods.

Previous work on analytical solutions of the time dependent equations of counter current systems with only solute exchange has been extended to give solutions for systems with differing ascending and descending flow velocities. These solutions have been compared with those obtained by numerical inversion of Laplace transforms.

A major effort has been directed toward establishing conditions for the existence and uniqueness of differential equations describing water and solute transport in flow systems. For a single tube exchanging water and a single solute with an external bath it has been possible to establish such conditions. It is anticipated that the analytical tools developed for the problem will be useful in studying more complicated flow equations.

The work on free-energy balance in renal counterflow systems has appeared (Math. Bioscience 21:299-310, 1974). This thermodynamic analysis has been extended to include the effects of viscous dissipation.

Project No. Z01 HL 03201-15 STB
1. ODIR
2. Section on Theoretical Biophysics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Mathematical Theory of Renal Function

Previous Serial Number: NHLI-239

Principal Investigator: John L. Stephenson, M.D.

Other Investigators: Raymond Mejia

Alan M. Weinstein (Student)
Harvard Medical School
Boston, Massachusetts

Bruce Kellogg, Ph.D.
Professor of Mathematics
IFDAM, University of Maryland
College Park, Maryland

Kenny Crump, Ph.D.
Professor of Mathematics
Louisiana Polytechnic Institute
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Jack Garner, Ph.D.
Professor of Mathematics
Louisiana Polytechnic Institute
Ruston, Louisiana

Cooperating Units: NIAMDD, Office of Mathematical Research

Institute of Fluid Dynamics and Applied Mathematics
University of Maryland

Department of Mathematics
Louisiana Polytechnic Institute
Ruston, Louisiana

Project Description:

Objectives: The primary purpose of this project is to develop the general theory of the transport and flow processes taking place in the kidney. This theory provides the general basis for quantitative models of renal function.

Major findings: 1) The thermodynamic analysis of flow through an isothermal system of tubes was extended (by Alan Weinstein) to include a

term representing dissipation of energy due to viscous flow. The dissipation per unit length was found to be approximately $R_i(F_{iv})^2$, where $R_i$ is the hydrodynamic flow resistance of the ith tube in the system and $F_{iv}$ is the axial volume flow. This energy dissipation was computed for a particular collection of models and its variation with changes in the parameters of the model studied.

2) Earlier work on analytical solutions of the time dependent equations of countercurrent systems with only solute exchange has been extended to give solutions for systems with differing ascending and descending flow velocities. These solutions have been compared with those obtained by numerical inversion of Laplace transforms (with J. Garner and K. Crump).

3) In a collaborative program with IFDAM, University of Maryland, a study was undertaken by Dr. Bruce Kellogg on the existence, uniqueness, and other mathematical properties of solutions of the differential equations describing kidney models. The main effort has been on the transport of fluid and solute in a single tube, with prescribed functions giving the transport of fluid and solute in or out of the tube.

One goal of the research was to determine conditions on these transport functions which guarantee the existence and uniqueness of a solution to the problem. In this study, a distinction was made between the equations with and without diffusion. The general conclusions of the study may be summarized as follows: For the problem with diffusion $D > 0$, a set of conditions were found which guarantee the existence and uniqueness of solutions. For the problem with $D = 0$, the solution may not exist. This is due to the possible presence of a "stagnant" point in the tube; that is, a point where flow becomes 0. A modified formulation of the boundary conditions was found with which the existence and uniqueness of solutions of the equations could be insured. This modified formulation gives rise to a boundary condition of an unusual kind, in that concentrations must be specified at both ends of the tube, provided the flow is entering both ends of the tube. The conditions on the fluid and solute fluxes that are required for the analysis of the equations without diffusion are somewhat more restrictive than was required for the equations with diffusion. Finally, the limiting behavior of the solution as D tends to 0 was studied. (This limiting process is known as a singular perturbation problem, and is frequently studied in other parts of applied mathematics.) It was found that, under appropriate hypotheses, the solutions of the problem with $D > 0$ converges to the solution with $D = 0$, thereby justifying the unusual boundary condition that was needed to analyze the presence of a stagnant point.

The main analytical tools used in this study were the method of continuation; that is, following a curve in a Banach space, and a modified form of the maximum principle that was used to establish the nonsingularity of the linearized problem. It is anticipated that these tools will be useful in studying more complicated flow equations.

4) Earlier work on kidney models was generalized to give equations for solute and water transport in an arbitrary network of exchanging flow tubes. The motivation for this was primarily economy in constructing algorithms describing a growing array of kidney models; but in developing a meta-theory for a hierarchical set of kidney models, it became obvious that except for specific vocabulary the theory described solute and water exchange in an arbitrary network of flow tubes in which fluxes, concentration, pressures and spatial configuration can be described by a suitably indexed set of functions, boundary conditions, and parameters. Such a description leads to a set of differential-integral equations for conservation of matter and the equations of motion. When combined with phenomenological equations for transmural flux, these equations can occasionally be solved analytically, but usuallly must be approximated by difference equations and solved numerically.

In this work we formulated this set of differential-integral equations and the approximating set of difference equations. A general scheme for solving these equations by the Newton-Raphson methods we have used for kidney models and a general theory of partitioning the equations were developed. This theory of partitioning is basic in the development of specific computational algorithms.

A concept which has emerged from this work is that from our point of view a model of a network of exchanging flow tubes is a set of difference equations that determine a state vector of the system $\Gamma_s$ implicitly as a function of a vector of boundary values $\Gamma_B$, and a vector of parameters $\Gamma_A$.

Symbolically we have

$$\phi_s(\Gamma_s, \Gamma_A, \Gamma_B) = 0, \qquad (1)$$

where $\phi_s$ denotes the set of equations.

In our modeling work to date we have regarded $\Gamma_s$ as unknown and solved the set of equations (1) for $\Gamma_s$ as a function of $\Gamma_B$ and $\Gamma_A$. The goal of this work has been to find a set of equations such that selected entries in $\Gamma_s$ correspond to experimental output data for given boundary conditions $\Gamma_B$, corresponding to experimental input data, and some reasonable set of parameters $\Gamma_A$. This type of simulation has reached the point where our models are behaving in a qualitatively reasonable way. We are now in a position to look at the inverse problem. Namely, given a set of experimental input output pairs, corresponding to selected entries in $\Gamma_s$ and $\Gamma_B$, to find $\Gamma_A$, so that input output pairs computed from the model will correspond as closely as possible. From this viewpoint each experimental input output pair $(\Gamma_s^e, \Gamma_B^e)$ will correspond to a model input output triple $(\Gamma_s^m, \Gamma_B^m, \Gamma_A^m)$. Within the limits of experimental error we can make experimental and model inputs identical, i.e.

$$\Gamma_B^m = \Gamma_B^e \qquad (2)$$

Each experiment will then yield a set of equations

$$\phi_e = \Gamma_s^e - \Gamma_s^m \qquad (3).$$

Ideally we seek to determine $\Gamma_A$ such that $\phi_e = 0$. Usually this is impossible and we must be content with minimizing $\phi_e$ with respect to some norm. Computationally such parameter evaluation is more difficult than simulation, and in the past would have been pointless because various models of the kidney and its subsystems failed on a qualitative level, but the present generation of models is beginning to permit quantitative comparison of model and experimental outputs.

Significance to biomedical research: This work gives conceptual insight into renal function and provides the basis for quantitative models of renal function.

Proposed Course: We plan to continue work in the following general areas:

1) The general theoretical analysis of the non-linear partial and ordinary differential equations occurring in mathematical models of the kidney.

2) The development of methods of parameter analysis for mathematical models of the kidney.

3) Thermodynamic analysis of models of the kidney.

Keyword
Descriptors: Mathematical theory of transport, thermodynamics, kidney, numerical inversion of Laplace transforms, viscous flow, differential equations, differential-integral equation, central core model of the kidney, mathematical model, solute and water transport in a network of exchanging flow tubes, membrane transport.

Honors and Awards: None.

Publications:

Stephenson, J. L. Free Energy Balance in Renal Counterflow Systems. Math Biosciences 21:299-310, 1974.

Project No. Z01 HL 03202-04 STB
1. ODIR
2. Section on Theoretical Biophysics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Computer Simulation of Renal Function

Previous Serial Number: NHLI-240

Principal Investigator: John L. Stephenson, M.D.

Other Investigators: Raymond Mejia

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SUNY
Stony Brook, L. I., New York

Walter Gross
Graduate Student
Department of Mathematics
University of Maryland

Project Description:

Objectives: The purpose of this project is to develop a computer simulation of the mammalian kidney that gives a realistic description of steady state and transient transport of electrolytes, non-electrolytes, and water. This will allow the correlation of micropuncture and macroscopic clearance data with membrane transport characteristics.

Methods: The numerical methods for solving the steady state and transient equations of the medullary counterflow system described in last year's report have been refined and extended. We now have computational methods that are sufficiently efficient to permit solution of models of the whole kidney. This work has included improvement of the Newton-Raphson methods by: 1) The development of specific sparse matrix routines (in collaboration with R. Tewarson, SUNY). 2) The adaptation of a general purpose sparse matrix algorithm. 3) The development of partitioning schemes that



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permit individual flow tubes to be solved against assumed interstitial concentrations and pressures. The interstitial concentration and pressure are then adjusted by Newton's method to give solute and water balance for each interstitial compartment. Solution of the equations for the individual tubes can either be a) by Newton's method, or b) a stepwise solution proceeding in the direction of flow. A comparative study has shown this last method to be accurate and the most efficient in the utilization of computer storage and time. Its extension to multinephron models of the whole kidney seems feasible.

Several quasi-linearization schemes for solving the time dependent equations of the kidney model have now been studied. (B. Hubbard and W. Gross.) The schemes are characterized by the fact that in advancing one time step, the solution of a linear system of equations is required; thus they give a potential improvement over fully implicit schemes, which require the solution of a set of nonlinear equations at each time step. The schemes can be used both to follow the changes in concentrations as a function of time, and to calculate the steady state values of the concentrations after the solution settles down.

These quasi-linear methods have been programmed for the three tube and six tube models of the medullary counterflow system, and various numerical experiments have been conducted. For sufficiently small time steps, all the schemes gave accurate results for the transient problem. As the time step increased, sooner or later all the schemes developed spurious oscillations. They differed considerably (by as much as a factor of 100) in the size of the time step where this occurred. The best scheme was found to be competitive with Newton's method in calculating the steady state solution.

Solution of the time dependent equations by numerical inversion of the Laplace transforms of the system has been extended (with K. Crump). The method should be very useful in the analysis of problems in which volume flow is not changing, e.g. isotope uptake and washout.

Major findings: The equations describing a model of the whole kidney were solved using a Newton-Raphson method. The method permits both steady state and transient solutions. With this model it has been possible to simulate behavior of the whole kidney as a function of hydrostatic pressures in renal artery, vein, and pelvis, protein concentration in arterial blood, and phenomenological equations describing transport of salt and water across nephron and capillary walls. Concentrations and hydrostatic pressures were computed for the various nephron segments and in cortical and medullary capillaries and interstitium.

In most ways calculations with the whole kidney model met intuitive expectations. Thus, increased proximal tubule delivery to the medulla increased urine flow and decreased urine concentration; increased proximal tubule and/or distal reabsorption increased glomerular filtration; autoregulation of the model could be obtained by adjusting afferent arteriolar resistance; and the behavior of the whole kidney model was in general agreement with models of the cortex or medulla alone.

New insights were obtained from the model with respect to the role of interstitial hydrostatic pressure. In the model this adjusts so as to give fluid balance in cortical and medullary interstitium. For this pressure to remain within reasonable physiological limits, hydraulic permeabilities of cortical and medullary capillaries must be greater than some critical minimum value.

We also have used our improved numerical methods to analyze more detailed models of the medullary counterflow system. These studies have confirmed our hypothesis that the central core model is the prototype for medullary function. In addition, they have suggested the probable importance of the cascaded structure of the medullary counterflow system. Calculations on a single nephron model of the medulla in which all nephrons are assumed to descend to the papilla have shown that enough urea is not available to drive the hypothesized passive salt transport system in the inner medulla. Calculations in a two stage model in which the urea entering the inner medulla via collecting duct reflects a 5/1 ratio of cortical to juxta medullary nephrons relieves this problem; but so far we have not been able to assign values to the various parameters in the model that will permit the inner medulla to concentrate passively and cycle the urea load observed at the loop. This has led us to hypothesize that urea concentration in ascending limbs of Henle that are returning from the depths of the inner medulla rises above urea concentration in the core, and urea diffuses out of these ascending loops and combine with urea supplied by the collecting duct to drive the passive mechanism for loops turning at an intermediate point. Equations have been formulated for this cascaded model, but we have not as yet made any detailed computations on it.

The studies on the whole kidney model and the medullary models have given further support to our thesis that the functional unit of the kidney is not the individual nephron, but a nephrovascular unit consisting of a group of nephrons and their tightly coupled vasculature.

Proposed Course: We have now developed our numerical methods to the point where it appears feasible to formulate and solve a multinephron multisolute model of the kidney that accounts for salt, water, and urea transport and hydrostatic and oncotic pressure. Simulation with this model should give us new insight into the role of urea in the concentrating mechanism and of the very intricate hydrostatic and oncotic pressure relationships in the kidney. In addition we believe that such a model will be sufficiently realistic to permit the first quantitative interpretation of overall renal function in terms of membrane transport characteristics.

Keyword
Descriptors: Computer simulation of renal function, kidney, transport of electrolytes, non-electrolytes and water, numerical methods, Newton-Raphson method, sparse matrix algorithms, quasi-linearization countercurrent system, hydrostatic and oncotic pressure, partitioning, flow processes, nephrovascular unit, steady state and transient transport.

Honors and Awards: None

Publications:

Stephenson, John L., R. P. Tewarson, and Raymond Mejia. Quantitative analysis of mass and energy balance in non-ideal models of the renal counterflow system. Proc. Nat. Acad. Sci. USA. 71:1618-1622, 1974.