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" Report of program activities

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ANNUAL PROJECT REPORTS

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INTRAMURAL RESEARCH

Dr. J. E. Rall, Director

ANNUAL REPORT SUMMARY

LABORATORY OF NUTRITION AND ENDOCRINOLOGY, 1972

PILOT PLANT UNIT

Large-scale Processing of Biological Materials

Reduction of some of the activities of the unit has been caused by the transfer of equipment from its current quarters in Building 3 to the new space in Building 6. Transfer of all equipment and full operation are expected by the fall of 1972.

During the past year, 200 requests by NIH investigators were processed. 153 kilogram quantities (43,000 liters of cultures) of 12 microorganisms were produced. The microorganisms grown during this period included protozoa, yeast and bacteria and consisted of regular and mutant strains of *Acanthamoeba castellanii, Bacillus subtilis, Escherichia coli, Hemophilus influenzae, Hemophilus parainfluenzae, Lactobacillus plantarum, Paramecium aurelia, Pseudomonas acidivorans, Pseudomonad Cre, Wg and MTC, Saccharomyces cerevisiae, Salmonella typhimurium and Streptococcus sp.* Group C. Some of the organisms were used as hosts for bacteriophage production e.g., Φ80 Lambda, MS-2, T4, and T7 and others, such as mutant strains of *Escherichia coli* were used to produce extracellular enzymes. Microorganisms were grown 96 times in the 300-liter fermentor, 23 times in the 10-liter, New Brunswick fermentor, 15 times in the 1100-liter fermentor and 34 times in the 50-liter Fermentation Design fermentor. Optimum growth conditions for maximum polysaccharide production by *Hemophilus influenzae* were studied during seven fermentations from 50 to 300 liters for the NICHD. Four 50-liter cultures of *E. coli*, one 50-liter culture of *Lactobacillus plantarum* and one 300-liter culture of *Streptococcus Sp.* Group C were processed for cross-reacting polysaccharides. Five attempts were made to prepare a heat-sensitive medium component from horse blood. *E. coli W6* spheroplasts were prepared three times using 10-, 50-, and 300-liter fermentors for each preparation. The Gaulin homogenizer was used 90 times to rupture suspensions of yeast or bacterial cells. Large-scale processing activities consisted of isolation of Hurler factor from human urine, histamine methylating enzyme from 1.5 kg guinea pig brains, special inhibitor from 21 kgm *Bacillus amyloliquefaciens*, histones from calf thymus, chromatin from rabbit liver, lipoprotein lipase from human plasma, red cell membranes from 20 units of human blood and partial processing of 300 lbs Baker's yeast for folates. The large capacity turbine Sharples centrifuges were used to separate cells from cultures such as 165 liters of *Hydrogenomonas utropha, Hemophilus influenzae* Rd and several phenolated cultures. Hydroxylapatite was prepared once in the 50-gallon Pfaudler reactor yielding 2 gallons of the chromatographic adsorbent. 150 lbs of animal diet and various quantities of plant and animal tissue were ground in the laboratory mills. Dialysis fluid and supernatant culture medium were concentrated *in vacuo* in the high capacity vacuum system. Twenty-five pounds of soy protein were washed with alcohol and dried in the explosion-proof area. (D. L. Rogerson Jr.)

GERM-FREE RESEARCH UNIT

Studies in Experimental Nutrition

Cycasin is not toxic to adult germfree rats but is toxic to conventional rats, to very young germfree rats and to ex-germfree rats infected with bacteria which are capable of converting the compound to its aglycone. The aglycone of cycasin is toxic to germfree as well as to conventional rats at any age. Although adult rats are incapable of converting cycasin to the aglycone in the absence of bacteria, the very young animal is able to do so, but this capability is lost early in life. Results at the present time indicate that, at some point between twenty-five and thirty-five days of age, the ability of the germfree rat to convert cycasin to the toxic aglycone is greatly reduced or delayed, if not completely lost. (G. Laqueur, M. Spatz (LEP,NIAMD) and E. G. McDaniel).

Experiments have been continued to study the effects of bacteria or "germ-freeness" upon iodine metabolism, thyroid function and the development of goiter. The effects of administration of sterile preparations of toxins of bacterial origin are also being studied. Changes observed appear to be related to the magnitude of inoculum as well as to the type of organism used. (R. L. Vought and E. G. McDaniel).

Germfree rats are able to survive for long periods without demonstrable amounts of vitamin A whereas conventional rats develop the typical symptoms of vitamin A deficiency and stop growing at about six weeks, then lose weight rapidly and die. Although germfree rats also reach a weight plateau and develop the typical symptoms of vitamin A deficiency, they continue to survive for periods up to a year. Early death in conventional rats was probably due to infection with specific types of bacteria. Experiments are being initiated to study the actual lesion caused by vitamin A deficiency and to identify the function of this vitamin at the cellular level. There are experiments in progress which are designed to examine any possible interrelationships between vitamin A and the trace element zinc. (J. Smith (VA Hospital), J. Bieri, W. Rogers and E. G. McDaniel).

It has been demonstrated that bacteria have a marked influence upon the development, the severity of deficiency symptoms, the survival time, and, probably upon the actual requirement for zinc. Germfree rats survived longer and exhibited less severe deficiency symptoms than conventional rats or ex-germfree rats which were inoculated with a normal bacterial flora. Experiments with $zinc^{65}$, given either by stomach tube or in the diet, indicate that although retention was effected by the degree of zinc deficiency, no difference in absorption or retention was observed. Early experiments were hampered by the occurrence of kidney stones in germfree rats. This has not been corrected by altering the levels of minerals in the diet. Increased levels of phosphorus have effectively prevented the kidney stones which have not been observed in animals fed quite similar diets based on unextracted casein.

Similarities in the symptoms of vitamin A and zinc deficiency were studied in double deficiency experiments, where it has been possible to precipitate a very early and acute vitamin A deficiency by treating this double deficiency with zinc. The syndrome is characterized by crippling which is more severe

and occurs much earlier than that resulting from accepted methods of vitamin A depletion. This phenomenon could be of clinical significance in cases where multiple deficiencies are present in a patient. It is known that deficiencies of zinc or vitamin A both have an effect on the sense of taste in humans and experiments are being initiated in rats to study the interaction of these two factors with respect to taste and smell. (J. Smith, (V. A. Hospital) and E. G. McDaniel).

Experiments are being continued to determine methods of altering the intestinal flora of animals in such a way as to have a beneficial effect upon a host which is subjected to diets of limited nutritional quality. With conventional animals, combinations of certain antibiotics or isolation along with antibiotics (life-island) has made it possible to maintain a bacterial flora in rats which permitted better utilization of inadequate diets. This provides basic information in planning human studies in areas where infant diseases of malnutrition occur. (F. S. Doft and E. G. McDaniel).

SECTION ON DEVELOPMENTAL BIOCHEMISTRY

Properties and Biological Activities of Oligonucleotides

Isolation of new trinucleotides is being accomplished by *Ustilago* ribonuclease digestion and aqueous methanol chromatography on the cellulose ion exchange adsorbents. Large-scale purification of RNase T1 and T2 has produced 4.1×10^5 A_{260} units of T1 with a specific activity of 8.4 and 1×10^3 A_{260} units of T2 with a specific activity of 8.5. (P. Roddy, G. W. Rushizky, and H. A. Sober).

Purification of the Hurler Corrective Factor

Hurler corrective factor is a protein absent in patients with a genetic disease of mucopolysaccharide metabolism known as the Hurler syndrome and which is characterized by excessive cellular accumulation of dematin and heparan sulfate. Urine and plasma were investigated as potential sources of the protein corrective factor, and it appears that urine is a richer source of this factor than is plasma, where it may exist in some cryptic form. Hurler factor has been purified from large pools of urine. Purification conditions are being worked out so that preparation of this factor for clinical use can eventually be handled by a contractor. (P. Roddy and E. Neufeld).

Studies on Structure and Function of Nucleic Acids

A procedure was developed for counting of ^{32}P-labeled RNA hydrolysates fractionated by a polyacrylamide gel electrophoresis, which involved strip chart counting with a gas flow counter, rather than slicing and counting in a scintillation counter. Because of difficulties encountered in growing ^{32}P-labeled bacterial viruses in liquid culture, a procedure on agar plates was developed. Several suitable bacterial viruses were examined as sources of homogeneous high molecular weight DNA. Φx 174 was found to be much more difficult to grow in large yields than the rod-like *E. coli* virus fd. DNA prepared from the latter will be used as a substrate for hydrolysis by various DNases.

Two-dimensional polyacrylamide gel electrophoresis of enzymatic digests

of MS2 RNA and fd DNA give reproducible patterns in 2-dimensions, as do f-met- and arginine tRNAs. Micrococcal nuclease, *B. amyloliquefaciens* RNase and *U. sphaerogena* RNase digests of MS2 RNA, were found to produce well-defined large oligonucleotide fractions of chain length comparable to that of tRNA. (G. W. Rushizky and H. A. Sober)

Structure and Function of Barnase and Barstar, an Extracellular Ribonuclease (Barnase) and its Intracellular Inhibitor (Barstar)

To further purify, characterize, and investigate this enzyme and its natural inhibitor by physical, chemical, and biological techniques, X-ray crystallographic studies have been performed at the Laboratory of Molecular Biology, Medical Research Council, Cambridge, England in collaboration with B. S. Hartley J. Sperling, D. M. Blow and R. C. Sheppard at the same laboratory.

Crystals of barnase suitable for X-ray crystallography may be grown in 30-40% saturated ammonium sulphate solution with the addition of zinc ions and β-mercaptoethanol. Neither of these additions affect the basic crystallographic structure, but do strongly affect nucleation and the relative growth of different crystal faces. The effect of mercaptoethanol is probably due to its sequestration of contaminating traces of heavy metal ions (e.g., Hg^{++}). Crystals so grown exhibit a high degree of order and very good stability in the X-ray beam. The molecules are packed as trimers into a trigonal lattice, giving nine molecules per unit cell. Additional symmetry within the three-molecule assymetric unit greatly improves the chances for a rapid solution of and structure.

Barnase, radioactively labeled at all residues and specifically labeled at the three phenylalanines, has been obtained. Strong evidence has been found, in the course of this work, that barnase as initially synthesized contains one less positive (or one more negative) charge than the purified material. (R. W. Hartley).

Fractionation of Chromatin and the Study of Nonhistone Proteins

Methods have been described for the fractionation of chromatin into its constituent species and for the partial subfractionation of the nonhistone proteins. With these methods, the nonhistones proteins were examined at different stages of differentiation to detect changes in the function of various cell types. Study of the mature and immature avian erythrocyte have led to the detection of only minor differences in nonhistone proteins between these two functional states of the cell, one actively synthesizing globin, and the other essentially inactive in transcription and protein synthesis.

Stimulation of guinea pig lymphocytes with phytohemagglutination leads to induction of marked metabolic activity, and, after a lag phase of about 24 hours, the onset of DNA synthesis followed by cell division. In collaboration with Drs. Ronald Levy and S. Rosenberg (NCI), the early changes in synthesis of histones and nonhistones in PHA-stimulated lymphocytes were studied in conjunction with the recently developed chromatin fractionation procedure. During the first hour after stimulation, there is only a very slight synthesis of histones, and this does not differ from that observed for the control unstimulated samples. In striking contrast, even at this early phase in the induction

of mitotic activity, a marked enhancement of synthesis of nonhistone proteins occurs consequent to the stimulation. The increased synthesis apparently involves all the protein groups detected by either charge- or size-based electrophoresis on polyacrylamide gels, or by chromatography on DEAE-cellulose, although the possibility of highly stimulated synthesis of a protein present in relatively small amounts can not be excluded. The changes documented by these studies occur in as short a time period after stimulation as any previously recorded for this system, and far precede the increase in synthesis of histones, RNA or DNA.

A third system, originally described by Shelton and Allfrey, showed that cortisol treatment of adrenalectomized rats led to preferential synthesis of a nonhistone protein of about 41,000 molecular weight. Preliminary studies have confirmed the greater degree of synthesis of the nonhistones as a class (vs. the histones) in treated animals. Attempts to isolate the protein observed by Shelton and Allfrey by our fractionation procedure are currently under way. (S. Levy, R. T. Simpson, and H. A. Sober).

Protein:Nucleic Acid Interaction - Chromatin Structure and Restriction in Eukaryotic Cells.

In continuing attempts to understand the topological relationships of DNA and proteins in chromatin, the binding of histones to DNA has been studied. For this purpose, the acetylation of chromatin with labeled acetic anhydride under controlled conditions (previously described) has been employed in order to modify only those lysyl residues of the histones which do not bind to nucleic acid in the native complex. Hence, isolation of the histones and determination of those lysyl residues in the protein primary sequence which are not modified, should indicate those regions of each histone which bind to DNA. Accordingly, histones were isolated from acetylated calf thymus chromatin and then digested with trypsin to produce peptides. Since acetyllysine is not a substrate for tryptic hydrolysis, longer peptides than those obtained in tryptic digestions of control histones were produced, with the length depending on the number of sequential lysyl residues modified for any given protein. Fractionation of the peptide digest led to isolation of six groups of labeled peptides, and demonstrated that, while most of the isotope was present in the larger peptides, there was little associated with the very small species. These size groups have then been further fractionated by sequential gradient chromatography on ion-exchange adsorbents. These procedures have led to the resolution of about 85-90 distinct peptides. Some 11-12 are radioactively labeled and should correspond to the nonbound regions of the five main histone fractions. Compositional and sequence studies on these peptides are currently underway to allow their localization in the known primary sequences of the various histones.

There has been some indication that chromatin possesses regions where the DNA has a single-stranded character, an important requirement of Crick's postulated model for chromosome structure. In attempts to ascertain the presence or absence, and amount, of a single-stranded DNA in chromatin using anti-single-strand DNA antibody induced in rabbits, quantitative complement fixation tests were performed with native and heat denatured chromatin, as well as controls of single and double stranded DNA, both alone and in their complexes with histones. It was demonstrated that there is not antigenically recognizable

single-stranded DNA in native chromatin (limit to detection less than 0.1%). These findings preclude this portion of the Crick model, and suggest that recognition in eukaryotic systems must either involve local unwinding of the double helix, or recognition of base sequences while the DNA is fully helical. (R. T. Simpson, and H. A. Sober).

Structural Analysis of the Active and Inactive Portions of the Eukaryotic Genome

As part of the effort to determine the physico-chemical basis for the functional differences between the transcribable portion and the remainder of the eukaryotic genome, sonication at 20,000 cycles per second has been used to reduce the size of the chromatin molecules and thus increase the possibility of having homogeneous populations. By melting profile analysis this somewhat drastic procedure produces, at most, only minimal effects on the chromatin beyond reduction in molecular weight.

Sonicated chromatin has been fractionated on ECTHAM-cellulose into a series of fractions having a continuous spectrum of melting profiles, the weak binding material melting at a considerably higher temperature than unfractionated chromatin and the latest eluting material having much more low melting component than unfractionated chromatin. Intermediate fractions melt essentially like chromatin. Although, there are not detectable quantitative differences in total protein content in the various fractions, there are qualitative differences revealed by gel electrophoresis. The tightly-bound material is strikingly enriched above unfractionated chromatin in a nonhistone of high molecular weight; in contrast, the weakly bound material lacks this protein. Roughly corresponding to the appearance of this protein is a decrease in the content of lysine-rich histone. No differences have been detected in the circular dichroic spectra of the fractions.

Attempts to determine which fractions contain the endogeneous RNA polymerase of chromatin have not been entirely successful, but preliminary indications are that the tightly bound material may contain this activity and hence be the transcribable portion of the genome. (G. R. Reeck, R. T. Simpson and H. A. Sober).

SECTION ON ENDOCRINOLOGY

Induction of Diabetes with Exogenous Hormones Modified by Tolbutamide

Studies of the hormonal induction of diabetes in normal rats, by growth hormone, ACTH, dexamethasone and cortisol have been extended to rats fed a diet containing 0.5% tolbutamide, an antidiabetic agent. Contrary to expectations, these rats responded to hormonal treatment by excretion of glucose in the urine on the second day of injection, whereas rats fed the regular diet and given the same hormone dosage developed glucosuria on the third or fourth day. When glucosuria on the fifth day was plotted against log-dose, as in a bioassay, the 0.5% tolbutamide diet was estimated to double the diabetogenic effect of the single hormones or their combinations.

The insulin concentration in blood and the blood sugar level were always greatly elevated on the fifth day in rats with hormonally induced glucosuria, while the insulin concentration in the pancreas was reduced with the development of glucosuria. The concentration of insulin in the pancreas however, tended to be lower in rats administered tolbutamide than in rats on the regular diet, suggesting greater depletion of the insulin reserve. (R. W. Bates).

Effect of Hormones on Adipose Tissue Perfused *in vivo*

Earlier studies in rat parametrial adipose tissue perfused *in vivo* showed that insulin injection stimulated FFA release in normal animals and suppressed FFA release in pancreatectomized rats. More recent studies showed that insulin also increased glycerol release in normal but not in pancreatectomized rats. These findings suggested that injection of insulin stimulated the secretion of lipolytic hormone by the pancreas. Results of earlier studies indicated that this hormone may not be glucagon.

Injection of growth hormone and dexamethasone increased FFA release *in vivo* from the parametrial fat pad in normal but not in adrenalectomized rats fasted for 1 day. ACTH, however, stimulated FFA, and also glycerol release, in both fasting normal and adrenalectomized rats. The molar ratio of FFA to glycerol released in response to ACTH was 3 in normal rats, and only 1 in adrenalectomized rats. This difference undoubtedly reflects a higher rate of reesterification of FFA by adipose tissue in adrenalectomized than in normal rats. This finding supports the hypothesis that glucocorticoids enhance FFA release by suppressing glucose utilization and FFA reesterification.

A method was developed for perfusing *in vivo* the epididymal fat body of the rat. This tissue also releases FFA in response to injections of TSH and epinephrine. (V. Kovacev, Univ. of Skopje, Yugoslavia, and S. S. Chernick).

Metabolism of Chylomicron-Triglyceride by Perfused Rat Mammary Gland

A technique for perfusing the inguinal-abdominal mammary tissue of lactating rats has been used to study the role of lipoprotein lipase in the uptake of chylomicron-triglyceride by mammary tissue. Perfused tissue of suckling rats retained in tissue lipid 10% of the chylomicron triglyceride-fatty acids infused and released 7% as FFA to the blood stream. The tissue also retained 9% of the chylomicron triglyceride-glycerol infused and released 6% as free glycerol. The molar ratio of FFA to glycerol released to blood was 15 during the first few minutes of chylomicron infusion, suggesting that partial glycerides were formed during the early stages of uptake. Non-suckling for 18 hours, which completely suppresses lipoprotein lipase activity in lactating mammary tissue, reduced by more than 90% both retention and hydrolysis of chylomicron-triglyceride. The findings show that hydrolysis occurs during the uptake of blood triglyceride by mammary tissue and that the rate of uptake is related to the level of activity of lipoprotein lipase in the tissue.
(Carole L. Mendelson and Robert O. Scow)

Metabolism of Chylomicrons and Lipoprotein Lipase Activity in Perfused Adipose Tissue

Earlier studies in perfused adipose tissue during uptake of chylomicron-

triglyceride showed that triglyceride was hydrolyzed within the capillary endothelium to FFA and glycerol. Two-thirds of the FFA were incorporated into tissue glyceride and the rest were released to the blood stream. More recent studies showed that the molar ratio of FFA to glycerol released to the blood was 5.1 during the first 30 seconds of infusion of chylomicrons and less than 1.4 during the rest of the infusion. These findings suggest that partial glycerides are formed during the first stage of uptake of blood triglyceride by adipose tissue. The following hypothesis, based on our recent biochemical and cytochemical studies, for transport of triglyceride-fatty acids across the capillary endothelium is now being tested. It is now proposed that soon after the chylomicron becomes attached to the luminal surface of the capillary endothelium, triglyceride in the chylomicron core is hydrolyzed by lipoprotein lipase to diglyceride and FFA. The diglyceride becomes attached to the enzyme bound to the endothelium plasma membrane and FFA is released to the blood stream. Vacuoles and microvesicles are then formed enclosing the diglyceride-enzyme complex and as they cross to the basal surface of the endothelium, diglyceride is hydrolyzed to monoglyceride and FFA. The contents of the vacuoles and microvesicles are then discharged to the basement membrane where the monoglyceride is hydrolyzed to FFA and glycerol; the FFA diffuse to fat cells and glycerol is released to the blood stream. (Margit Hamosh, E. Joan Blanchette-Mackie, Anthony J. Evans and Robert O. Scow)

Lingual Lipase and Digestion

A very potent lipolytic activity was recently discovered in rat tongue during a search for the source of lipolytic activity found in the stomach contents of suckling rats. Further studies have shown that the activity is present mainly in lingual serous glands located in the posterior part of the tongue, and that the activity is secreted into the oral cavity through ducts which open into the furrows of the circumvallate and the folliate papillae. The enzyme activity in the tongue, of both suckling and adult rats, hydrolyzes triglyceride to diglyceride, monoglyceride, and FFA; about 2/3 of the glycerides formed are diglyceride and the rest are monoglyceride. The pH optimum of the reaction is 4.5-5.5. The enzyme activity is also found in saliva and gastric contents of rats fed 50% cream in milk. The activity in gastric contents, however, is negligible when the meal is given by stomach tube and saliva is excluded from the stomach. Examination of several species (rat, calf, dog, man) showed that FFA accounts for about 1/3 of the fatty acids present in the stomach after a fat meal and that the pH of gastric contents during digestion is 4.5 - 5.5.

These studies show that the serous glands of tongue secrete a potent enzyme, lingual lipase, which can act in the stomach to break down triglyceride to partial glycerides and FFA. It is suggested that this action of the enzyme facilitates emulsification of dietary lipid before it enters the duodenum. (Margit Hamosh and Robert O. Scow)

Hormonal Control of Lipoprotein Lipase Activity in Mammary and Adipose Tissue During Lactation

Previous studies in this laboratory showed that lipoprotein lipase activity is increased in mammary tissue and decreased in adipose tissue during lactation. When suckling was stopped, lipolytic activity decreased in mammary tissue and

increased in adipose tissue. Recent studies in hypophysectomized lactating rats, given various combinations of hormones needed for maintenance of lactation, showed that prolactin alone increases lipoprotein lipase activity in mammary tissue and suppresses the activity in adipose tissue; the effects of prolactin were enhanced slightly by concurrent injection of dexamethasone or of dexamethasone, growth hormone, and thyroxine. Prolactin had no effect on lipoprotein lipase activity in nulliparous animals. (Oren Zinder and Robert O. Scow)

Effect of Lipoprotein Lipase on the Structure of Chylomicrons

Chylomicrons are spheres of triglyceride enveloped by a monomolecular surface coat of phospholipid, cholesterol, and protein. Studies using the transmission and scanning electron microscope have shown that the surface becomes wrinkled when the triglyceride content of the chylomicrons is decreased by the action of lipoprotein lipase, indicating that the surface coat is pliable and resistant to the action of the enzyme. (E. Joan Blanchette-Mackie and Robert O. Scow)

SECTION ON MEMBRANE REGULATION

Regulation of Adenylate Cyclase Systems

Adenylate cyclase systems in animal cells are localized in the membrane and are stimulated by a variety of peptide hormones, biogenic amines and prostaglandins. These systems are composed of a regulatory component (or receptor) and adenylate cyclase, the enzyme that converts ATP to cyclic AMP. The hepatic enzyme system of the rat responds to glucagon which binds to the regulatory component through reversible, non-covalent forces. In this system, the regulatory component is linked to the enzyme in a manner such that glucagon binding results in rapidly reversible changes in enzyme activity when the substrate (ATP) is provided at concentrations higher than 10^{-4} M. At lower substrate concentrations, no hormone effect is observed unless either GTP or GDP (10^{-8} to 10^{-6} M) is present. Since the guanyl nucleotides are not substrates for the enzyme, it is assumed that they act at some site other than the catalytic site. Similar actions of GTP have been observed on the glucagon-sensitive system in the pancreas beta cell and on the prostaglandin-sensitive system of human platelets.

The site of action of the nucleotides remains unknown but their actions on the hepatic system result in an increased rate of glucagon dissociation from the regulatory component without changing the rate of association. This change in the glucagon affinity of the regulatory component is considered to reflect an alteration, due to nucleotide binding, in the "coupling" of the regulator and catalytic components.

Phospholipids appear to be involved in glucagon binding, coupling of regulatory and catalytic components, and in enzyme activity. Treatment with detergents and phospholipase A results in a loss of both binding and activity of glucagon, but an increase in the response of the catalytic component to fluoride ion, which enhances the activity of all adenylate cyclase systems even when the regulatory components have been removed by proteolytic digestion

or by solubilization of the enzyme. Phospholipase C from *B. cereus*, which catalyzes the hydrolysis of all phospholipids in liver membranes except sphingomyelin, inhibits the hepatic system response to glucagon without loss of the fluoride response. In contrast, phospholipase C from *Cl. perfingens*, which catalyzes the hydrolysis of sphingomyelin, lecithin, phosphatidylethanolamine, but not acidic phospholipids (phosphatidylserine or phosphatidylinositol), only slightly reduces the response to glucagon or fluoride ion even though the percentage of phospholipids hydrolyzed in the hepatic membrane by both phospholipases is the same. Such findings suggest that acidic phospholipids, present in only small amounts in the membrane, may be specifically involved in the processes through which glucagon sitmulates the enzyme system.

In contrast to the hepatic system, the adenylate cyclase system in fat cells of the rat consists of several regulatory components each specific for epinephrine, ACTH, secretin, glucagon, LH, and TSH. These components are linked to a single catalytic component. As with the hepatic system, treatment with detergents and phospholipase A results in loss of all hormone responses but in retention or enhancement of the fluoride response.

Unlike the glucagon or prostaglandin-stimulated systems described above, the fat cell system responds fully to epinephrine in the presence of very low concentrations of ATP or AMP-PNP (a substrate for adenylate cyclase that is not hydrolyzed at its terminal phosphate). Addition of GTP results in marked inhibition of epinephrine stimulation; an effect not observed with GDP and lost if the concentration of GTP is not maintained during incubation. Basal activity (no hormonal addition) and fluoride response are also inhibited by GTP which suggests that the nucleotide modifies the catalytic component, possibly through phosphorylation. On the other hand, either GTP or GDP at low concentrations markedly enhance the response of the fat cell system to ACTH and glucagon, and cause moderate increases in its response to secretin. The equivalent effects of low (10^{-7}M) concentrations of GTP and GDP on the adenylate cyclase response to peptide hormones suggest that they act by binding to sites involved in the coupling of regulatory and catalytic components.

Calcium ion is required specifically for the action of ACTH on the fat cell system; its removal by chelators results in loss of ACTH response with loss of response to any of the other hormones. Neither GTP or GDP prevent the loss of ACTH response, indicating that calcium ions and the nucleotides act at distinct sites involved in ACTH action. The calcium site is probably not related to binding of ACTH to the regulatory component since other investigators have shown that calcium ion is not required for ACTH binding to its receptor.

The specific effects of GTP on inhibition of the epinephrine response, the differing effects of GDP on the response of the adenylate-cyclase system to the peptide hormones, and the specific requirement of the ACTH response for calcium ions suggest that, though the regulatory components for these hormones interact with a common catalytic component, the coupling of these components to the enzymes may be quite different. Coupling may not depend simply upon the presumed change in conformation of the regulatory component induced by hormone binding; the conformational state of the catalytic component and factors, such as phospholipids and nucleotides, appear to be fundamental to the

coupling and ultimate activation of the enzyme.

Studies are in progress to determine whether the inhibition of the epinephrine response by GTP is the result of a phosphorylation process, and, if so, whether this process is regulated through a separate enzyme in the plasma membrane. Such studies may reveal other regulatory processes in the plasma membrane which are involved in the regulation of adenylate cyclase and its response to hormones. (Martin Rodbell, James Harwood, Hans Low, Dean Londos, Boanerges Rubalcava and Michael Lin).

SECTION ON NUTRITIONAL BIOCHEMISTRY

Metabolism and Function of Fat-soluble Vitamins

Studies comparing the metabolism of α- and γ-tocopherols in the rat have shown that the two compounds are absorbed from the intestine unchanged and are distributed similarly in the lymph lipoproteins. When injected intravenously in lymph, γ-tocopherol disappeared from the plasma and tissues faster than did α-tocopherol. Plasma α-tocopherol levels in monkeys fell rapidly when vitamin E was removed from the diet. The dietary requirement for vitamin E for rhesus monkeys was estimated to be 5 mg/kg and twice this was an adequate allowance. Analysis of representative U.S. human diets showed that the daily intake ranges from 4 to 12 mg of α-tocopherol. (J. G. Bieri, I. R. Peake and R. Poukka Evarts).

Biochemical Studies of Cytochromes

Continuing studies of sub-cellular particles present in mitochondria indicate that these particles possess many attributes of conventional mitochondria as indicated by enzyme markers and measures of integrity of the particles. However, on density gradient centrifugation they band sharply at a significantly lower density than conventional mitochondria. The particles also have certain attributes of microsomes as measured by enzyme markers, some of which peak with the particles and some of which do not in sucrose density gradients. Contaminants of preparations of these particles are lysosomes and peroxisomes, both of which, however, can be separated on density gradients from the particles in question. The lysosomal and peroxisomal contaminants exhibit biphasic peaks in the gradients. (J. N. Williams, Jr.)

Synthesis and Metabolism of Plasma Lipids and Lipoproteins

Several aspects of lipid and lipoprotein metabolism were investigated in intact rats with mesenteric lymph and bile fistulae and in isolated, hemoperfused rat intestine and liver. Synthesis *de novo* of fatty acids by intestine was quantitated *in vivo* and in the perfused organ by using $^{3}H_{2}O$ as precursor. Compared with liver and adipose tissue, the synthetic rate in the gut was found to be low. Synthesis of the various apoproteins of plasma lipoproteins was measured in isolated perfused liver and intestine. Liver synthesized all the known apoproteins, while the intestine synthesized only some of them. The results also indicated that nascent lipoproteins synthesized by liver and intestine are altered by combining with lipoproteins already in the circulation. In another study, the turnover and metabolism of plasma

lipoproteins in rats was studied after intravenous injection of ^{125}I-labeled rat and human lipoproteins. Evidence was obtained for a divergence in the metabolism of the several apoproteins of plasma high density lipoproteins. (H. G. Windmueller).

SECTION ON VITAMIN METABOLISM

Studies on Folic Acid

Dihydrofolic reductase from yeast has been purified approximately 10,000-fold. However, the material is far from homogenous, and quite unstable, especially at high dilution or low ionic strength. Kinetic analysis of the mercury-treated chicken liver dihydrofolic reductase suggests that dihydrofolate must be present at approximately 2-3 times this K_m concentration (7×10^{-7}M) in order for the activated rate to be evident. TPNH also plays a role in this process which, however, is more complex and may be involved in maintaining the activated conformation.

The complex folate isolated from yeast appears to have the empirical formula: $C_{50}H_{58}N_{13}O_{24}Ba_4 \cdot 8H_2O$ which is consistent with a heptaglutamate of N-5-methyltetrahydrofolate. Elemental analysis suggest this product is now 98% pure. (B. T. Kaufman).

Properties of Folic Acid γ-Glutamyl Carboxypeptidases.

To purify folic acid γ-glutamyl carboxypeptidase (conjugase), chicken pancreas, either fresh or as acetone powder, was used because of its high level of conjugase activity and its relatively cheap and ready supply. A stable enzyme preparation was achieved by removal of trypsin and chymotrypsin or their zymogens either by affinity chromatography or gel filtration. Having obtained a stable preparation and characterized the behavior of the enzyme by ammonium sulfate precipitation, DEAE-cellulose chromatography, gel filtration and several potential affinity chromatographic systems, final purification is under way.

To facilitate this task a rapid assay has been developed to supplement the time consuming microbiological technique which has been in use for years. The substrate used is a yeast folic acid derivative with seven glutamyl residues, most of which are hydrolyzed during conjugase action to give a product having one or two glutamyl residues. The concomitant reduction in negative charge from the heptaglutamate to the product of conjugase digestion should allow separation by ion exchange chromatography.

The very sensitive and rapid technique of DEAE-cellulose thin-layer chromatography is used for the analysis of conjugase reaction mixtures and provided good separation of starting material and product, eliminating the requisite overnight incubation and considerable manipulation of the microbiological assay. In addition to its utility in assaying for conjugase, the thin layer system, will provide a valuable tool in assessing the state of oxidation, ring substitution and glutamic acid content of naturally occurring and synthetic folates. (G. R. Reeck and H. Bakerman)

COLLABORATIVE RESEARCH

Effects of Hormone Metabolism of Adipose Tissue Perfused *in vivo*:-
with Dr. Vance Kovacev, Department of Physiology, Medical Faculty, University of Skopje, Skopje, Macedonia, Yugoslavia (S. S. Chernick)

Metabolic Studies of Vitamin A:-
with Dr. J. Ganguly, Department of Biochemistry, Indian Institute of Science, Bangalore, India (J. Bieri)

Control of Lipid Synthesis by the Liver:-
with Dr. Benyamin Shapiro, Department of Biochemistry, Hebrew University, Hadassah Medical School, Jerusalem, Israel (M. Rodbell)

Effect of DNA and DNA Nucleotides in the Reaction of the Immune Response:-
with Dr. Michael Feldman, Department of Cell Biology, Weizmann Institute of Science, Rehovoth, Israel (H. A. Sober)

A Study of Individual Oligopeptide Members of Polyamino Acid Series:-
with Dr. Ephraim Katchalski, Department of Biophysics, Weizmann Institute of Science, Rehovoth, Israel (H. A. Sober)

Serial No. NIAMD-LNE-1
1. Nutrition and Endocrinology
2. Vitamin Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on folic acid.

Previous Serial Number: SAME

Principal Investigator: Dr. B. T. Kaufman

Other Investigators: Miss R. C. Gardiner and Mrs. M. K. Romine

Cooperating Units: None

Man Years

Total:	4.0
Professional:	3.0
Other:	1.0

Project Description:

Objectives: To isolate and determine the biochemical properties of enzymes concerned with the metabolism of folic acid especially dihydrofolic reductase from chicken liver, yeast, *Salmonella typhimurium*, *Escherichia coli*, and certain tumor cells. To isolate and determine the chemical and biological properties of the naturally occurring forms (polyglutamates?) of folic acid in microbial and animal cells.

Methods Employed: Standard chemical and biochemical techniques appropriate for purification and fractionation of vitamins and proteins are utilized in these projects in conjunction with various methods of enzymatic and microbiological analyses.

Major Findings: Dihydrofolic reductase: (A) Yeast Enzyme: Dihydrofolic reductase from yeast has been purified approximately 10,000-fold by a combination of affinity chromatography, hydroxylapatite chromatography and gel filtration. Despite this high degree of purification, this preparation appears to be only 10% pure. The specific activity of the yeast enzyme appears to be much higher than the chicken liver enzyme. Preliminary studies suggest that the marked lability of this enzyme is due to dissociation into inactive subunits at high dilution or exposure to low ionic strength. The inactive preparation may be reactivated by preincubation with high concentrations of thiol compounds.

(B) Chicken Liver Enzyme: Kinetic analysis of the chicken liver dihydrofolic reductase suggests that the activation of the enzyme resulting from

treatment with methyl mercuric hydroxide depends on the binding of dihydrofolate. In other words, the mercury-enzyme as such is not the activated species. This analysis indicates that dihydrofolate must be present at a concentration approximately 2-3 times its K_m (7×10^{-7}M) value for the stimulated activity to become evident, despite an excess of TPNH. At dihydrofolate concentrations less than 7×10^{-7}M, the mercury enzyme exhibits no activation as compared to the untreated enzyme. Similarly, when folate is used as the substrate, activation of the reaction rate is observed only when folate concentration is again greater than 2-3 times its K_m concentration (6×10^{-6}M). This suggests that the binding of folate may also induce the activation. However, due to the nature of its binding, a concentration approximately 10-fold higher than dihydrofolate is required. On the other hand, dihydrofolate may be the activating agent in this case also and this increased folate concentration is required to achieve the required level of dihydrofolate in the steady state of the reaction.

Although this study does not suggest a role for TPNH in this process, the marked differences in the kinetic properties of the mercury-treated enzymes vs the native enzyme when TPNH and DPNH are used as reducing agents require an additional role for TPNH. For example, activation is observed at all concentrations of TPNH when dihydrofolate is present in excess. However, activation is only observed when the DPNH concentration is less than 3×10^{-4}M, i.e., less than saturation - despite an excess of dihydrofolate. In order to explain these results it is postulated that TPNH is capable of maintaining the activated conformation once induced. Thus, when the steady state concentrations of dihydrofolate drops below this critical concentration due to the very rapid rate of the reaction occurring at very high concentrations of the reduced pyridine nucleotide, the activated state persists. However, DPNH which cannot protect the enzyme from heat denaturation or troteolytic digestion (in a manner similar to TPNH), does not bind in a way to maintain this activated conformation.

Naturally Occurring Forms of Folic Acid: The water soluble barium salt of the complex folate found in yeast yielded the empirical formula

$$C_{50}H_{58}N_{13}O_{24}Ba_4 \cdot 8H_2O$$

based on the assumption that it is a derivative of N-5-Methyl-tetrahydrofolate and containing a chain of seven glutamic acids plus the following analytical data:

C	H	N	Ba	
30.93	3.83	9.36	29.24	Found
31.29	3.88	9.49	28.63	Calc.

This suggests that this material is at least 98% pure.

Significance to Bio-medical Research and the Program of the Institute: Dihydrofolic reductase is not only a key enzyme in the metabolism of folic acid, it is also of major importance in the synthesis of thymidine and the chief site of action of the anti-folic drugs. Thus, the structure and function of this enzyme would be of basic importance in both the understanding of normal and metabolic interrelationships and cancer chemotherapy. Folic acid as such

most probably does not occur in nature. The knowledge of chemical structure and biological properties of the more complex natural form will result in a better understanding of the biochemistry of this vitamin.

Proposed Course of Project: Investigations will continue on the properties and mechanism of activation of chicken liver dihydrofolic reductase. The lack of similar activations of the enzymes derived from yeast and bacteria will be examined in relation to the properties of this enzyme. This will require the isolation of dihydrofolic reductase from these microbial sources. The current studies on the yeast folate are being directed twoard elucidation of its structure and biochemical function.

Honors and Awards: NONE

Publications:

Kaufman, B. T.: Isoelectric focusing studies on dihydrofolic reducatase. Ann. of the N.Y. Acad. of Sci., 186: 100-106, 1971.

Kaufman, B. T. and Pierce, J. V.: Purification of dihydrofolic reductase from chicken liver by affinity chromatography. Biochem. Biophys. Res. Commun. 44: 608-613, 1971.

Serial No. NIAMD-LNE-2
1. Nutrition and Endocrinology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Large-scale processing of biological materials.

Previous Serial Number: SAME

Principal Investigator: Mr. D. L. Rogerson, Jr.

Other Investigators: Dr. H. A. Sober

Cooperating Units: None

Man Years

Total:	3.0
Professional:	1.0
Other:	2.0

Project Description:

Objectives: Large amounts of natural materials, such as bacteria, animal tissues, cells, blood, urine and plant materials require facilities for their production and processing so that they may be studied under standard laboratory conditions. Facilities for such large-scale processing are provided under this project.

Methods Employed: When called upon for large-scale production by any NIH investigator, methods are designed for the scaling up of small laboratory experiments utilizing large reaction vessels, extractors, stills, concentrators, centrifuges, etc., so that the processing can be carried out effectively, efficiently and safely. Two 10-liter, one 50-liter, two 100-gallon and one 300-gallon fermentor are utilized for growing microorganisms under a variety of conditions. The bacterial cells and yeasts are harvested by means of laboratory or large-scale AS-16P centrifuges. Protozoa are concentrated in continuous flow centrifuges. Cells are either supplied as a paste or further processed to yield cell-free suspensions by rupture with a Gaulin homogenizer. In some instances a specific substance is isolated and partially purified by further processing of the culture supernatant or the cell paste. A high capacity, Model AS-26P Sharples centrifuge is now on order to facilitate the recovery of microorganisms from the 1100-liter fermentor.

Major Findings: During the past eleven months, 200 requests by NIH investigators were processed. 153 kilogram quantities (43,000 liters of cultures) of 12 microorganisms were produced. The microorganisms grown during this period included protozoa, yeast and bacteria and consisted of regular

and mutant strains of *Acanthamoeba castellanii, Bacillus subtilis, Escherichia coli, Hemophilus influenzae, Hemophilus parainfluenzae, Lactobacillus plantarum, Paramecium aurelia, Pseudomonas acidivorans, Pseudomonad Cre, Wg* and *MTC, Saccharomyces cerevisiae, Salmonella typhimurium* and *Streptococcus sp.* Group C. Some of the organisms were used as hosts for bacteriophage production such as Φ80, Lambda, MS-2 T_4, and T_7 and others, such as mutant strains of *Escherichia coli* were used to produce extracellular enzymes. Microorganisms were grown 96 times in the 300-liter fermentor, 23 times in the 10-liter New Brunswick fermentor, 15 times in the 1100-liter fermentor and 34 times in the 50-liter Fermentation Design fermentor. Optimum growth conditions for maximum polysaccharide production by *Hemophilus influenzae* were studied during seven fermentations from 50-300 liters for the NICHD. Also four 50 liter cultures of *E. coli,* one 50-liter culture of *Lactobacillus plantarum* and one 300-liter culture of *Streptococcus sp.* Group C were processed for cross-reacting polysaccharides, and five attempts were made to prepare a heat-sensitive medium component from horse blood. *E. coli W6* spheroplasts were prepared three times using 10-, 50-, and 300-liter fermentors for each preparation. The Gaulin homogenizer was used 90 times to rupture suspensions of yeast or bacterial cells. Large-scale processing activities consisted of isolation of Hurler factor from human urine, histamine methylating enzyme from 1.5 kgm guinea pig brains, special inhibitor from 21 kgm *Bacillus amyloliquefaciens,* histones from calf thymus, chromatin from rabbit liver, lipoprotein lipase from human plasma, red cell membranes from 20 units of human blood and partial processing of 300 lbs Baker's yeast for folates. The large capacity and turbine Sharples centrifuges were used to separate cells from cultures such as 165 liters *Hydrogenomonas utropha, Hemophilus influenzae* Rd and several phenolated cultures. Hydroxylapatite was prepared once in the 50-gallon Pfaudler reactor yielding 2 gallons of the chromatographic adsorbents. 160 lbs of animal diet and various quantities of plant and animal tissues were ground in the laboratory mills. Dialysis fluid and supernatant culture medium were concentrated *in vacuo* in the high capacity vacuum system. Twenty five pounds of soy protein were washed with alcohol and dried in the explosion-proof area.

Significance to Bio-Medical Research and the Program of the Institute: With the laboratory's facilities many projects can be assisted where large amounts of natural material can be produced and processed.

Proposed Course of Project: To continue assisting NIH investigators when large-scale processing of biological materials is needed in their projects and to expand the facilities for isolation and purification of biologically-important macromolecules, cell particulates and materials.

Honors and Awards: None

Publicat-ons:

Hartley, R. W., and Rogerson, D. L. Jr.,: Production and purification of the extra-cellular ribonuclease of Bacillus amyloliquefaciens (Barnase) and its intra-cellular inhibitor (Barstar). I. BARNASE. Preparative Biochemistry (in press)

Hartley, R. W., and Rogerson, D. L. Jr.,: Production and purification of the extra-cellular ribonuclease of *Bacillus amyloliquefaciens* (Barnase) and its intra-cellular inhibitor (Barstar): II. BARSTAR. Preparative Biochemistry, (in press)

Rechler, M. M., Bruni, C. B., Martin, R. G., Guyer, R., Poy, G., Rogerson, D., and Terry, W.: An intercistronic region in the histidine operon of *Salmonella typhimurium*. II. Mechanism of discoordinate polarity in HisD2353. J. Mol. Biol. (in press)

Serial Number NIAMD-LNE-3
1. Nutrition and Endocrinology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies in experimental nutrition.

Previous Serial Number: SAME

Principal Investigator: Mr. E. G. McDaniel

Other Investigators:
Dr. F. S. Doft, Guest Worker (LNE-NIAMD)
Dr. R. L. Vought, (EFS-NIAMD)
Dr. G. Laqueur, M. Spatz (LEP-NIAMD)
Dr. J. Bieri (LNE-NIAMD)
Dr. W. Rogers, NIDR
Dr. J. Smith, V. A. Hospital, Trace Element Lab.

Cooperating Units: NONE

Man Years

Total:	3.0
Professional:	1.0
Other:	2.0

Project Description:

Objectives: To determine the nutritional, biochemical and physiological characteristics of germfree and conventional laboratory animals.

Methods Employed: Germfree and conventional rats and chicks are maintained on diets of known composition and growth. Blood picture and other physiological functions are studied. Germfree animals are inoculated with known strains of bacterium to determine the organisms responsible for various phenomena. Conventional and/or ex-germfree animals are used in conjunction with germfree work.

Major Findings: With Dr. Laqueur and Dr. Spatz, studies of the toxic effect of cycasin have been continued. Cycasin is not toxic to adult germfree rats but is toxic to conventional rats, to very young germfree rats and to ex-germfree rats infected with bacteria which are capable of converting the compound to its aglycone. Previously germfree rats, infected with bacteria which are unable to make this conversion, react like germfree rats and show no symptoms of toxicity to cycasin. The aglycone of cycasin is toxic to germfree as well as conventional rats at any age. Although adult rats are incapable of converting cycasin to aglycone in the absence of bacteria, the very young animal is able to do so but this capability is lost early in life. Experiments

in progress are designed to determine at what period the animal loses the ability to make this conversion within its own tissue and without the presence of bacteria. Single doses of cycasin were administered to germ free rats at five, nine, sixteen, twenty-five and thirty-five days of age. Results at the present time indicate that at some point between twenty-five and thirty-five days of age, the ability of the germfree rat to convert cycasin to the toxic aglycone is greatly reduced or delayed, if not completely lost.

With Dr. Vought, experiments have been continued to study the effects of bacteria of "germfreeness" upon iodine metabolism, thyroid function and the development of goiter. Methods studied to effect an alteration of the intestinal flora of rats include use of antibiotics, monoinfection or controlled infection of previously germfree rats with various types of bacteria, "over-infection" of conventional animals and isolation. The effects of adminstration of sterile preparations of toxins of bacterial origin are also being studied. Changes observed appear to be related to the magnitude of inoculum as well as to the type of organism used.

With Dr. Bieri and Dr. Rogers, the study of vitamin A deficiency in rats is being continued. Germfree rats are able to survive for long periods without demonstrable amounts of vitamin A compared to conventional and ex-germfree rats fed similar diets. Conventional rats develop the typical symptoms of vitamin A deficiency and stop growing at about six weeks, then lose weight rapidly and die. Although germfree rats also reach a weight plateau and develop the typical symptoms of vitamin A deficiency, they continue to survive for periods up to a year. There was evidence that the early death in conventional rats was due at least in part to infection. It was indicated that the early death was probably the result of infection with specific types of bacteria but not all bacteria. Experiments are being initiated to study the actual lesion caused by a deficiency of vitamin A, and to identify, if possible, the function of this vitamin at the cellular level. There are experiments in progress which are designed to examine any possible interrelationship between vitamin A and the trace element zinc.

With Dr. Smith, a series of experiments have been conducted to determine whether the requirements for, or the deficiency symptoms of the trace element zinc are effected by intestinal bacteria. It has been demonstrated that bacteria do have a marked influence upon development, severity of deficiency symptoms, survival time, and probably upon the actual requirement for zinc. Germfree rats survived longer and exhibited less severe deficiency symptoms compared to conventional rats or rats which had been germfree but were inoculated with a normal bacterial flora. Although still evident, the difference between germfree and conventional rats became less marked as lower and lower levels of dietary zinc were attained. This suggests that at least part of the differences observed between germfree and conventional rats may be due to a more efficient utilization of low amounts of zinc in the absence of bacteria. In order to gain information regarding the influence of bacteria on absorption and/or retention of zinc, experiments were done in which germfree and non-germfree rats in various stages of zinc depletion, were given $zinc^{65}$ either by stomach tube or in the diet. Results indicate that although retention was

effected by the degree of zinc deficiency, no difference was observed between germfree and non-germfree animals.

Early experiments were hampered by the occurrence of kidney stones in germfree rats. This was at first thought to be the result of zinc deficiency, but was then shown to occur even with zinc supplementation. This difficulty appears now to have been corrected by altering the minerals in the diet. Increased levels of phosphorus have effectively prevented the kidney stones. It is possible that, during extraction of casein to lower the zinc content, phosphorus and perhaps other factors were also removed. Kidney stones have not been observed in this laboratory in animals fed quite similar diets in which unextracted casein is used.

There are some similarities in the symptoms of vitamin A deficiency and zinc deficiency. To study any possible interrelationship between these two factors, rats have been subjected to a double deficiency. It has been possible to precipitate what appears to be a very early and acute vitamin A deficiency by treating this double deficiency with zinc. The apparent vitamin A deficiency is characterized by crippling which is much more severe and occurs much earlier than by accepted methods of vitamin A depletion. Whether this observation indicates a relationship between zinc and vitamin A remains to be determined. Experiments are in progress to study the mechanism by which this abnormally rapid onset of vitamin A deficiency is accomplished. This phenomenon could be of clinical significance in cases where multiple deficiencies are present in a patient. It is known that deficiencies of zinc or vitamin A both have an effect on the sense of taste in humans. Experiments are being initiated to study this in rats and to determine if there is any interaction of these two factors with respect to taste and smell.

With Dr. Doft, experiments are being continued to determine methods of altering the intestinal flora of animals in such a way as to have a beneficial effect upon a host which is subjected to diets of liminted nutritional quality. It has been found that with conventional animals and combinations of certain antibiotics or isolation along with antibiotics (life-island), it is possible to maintain a bacterial flora in rats which does permit better utilization of inadequate diets. This provides basic information in planning human studies in an area where infant diseases of malnutrition occur.

Significance to Bio-Medical Research and the Program of the Institute: The work with germfree animals will give us a better understanding of the relationship between the animal's intestinal flora and its physiological reactions both to dietary nutrients and to other factors. The work with the germfree animals emphasizes the importance of nutrition in long-term studies.

Proposed Course of Project: Experiments will be conducted in an attempt to determine more precisely the relationship between the host animal and its bacterial flora with respect to its influence upon the nutrition and physiology of the host. Attempts will be made to identify the specific organisms which are responsible for such influence and, if possible, determine methods to counteract such influences as may be deleterious to the host.

Honors and Awards: NONE

Publications:

Vought, R. L., Brown, F. A., Sibinovic, K. H. and McDaniel, E. G.: Effect of changing intestinal bacterial flora on thyroid function in the rat. Hormone and Metabolism Research, 4: 43-47, 1972.

Smith, J. C. Jr., McDaniel, E. G., McBean, L. D., Doft, F. S., and Halstead, J. A.: Effect of micro-organisms upon zinc metabolism using germfree and conventional rats. J. of Nutr. (in press)

Smith, J. C. Jr., and McDaniel, E. G.: Increased urolithiasis in germfree rats. Investigative Urology. (in press)

Serial No. NIAMD-LNE-4
1. Nutrition and Endocrinology
2. Endocrinology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Study of protein hormones: Chemistry and physiological actions of protein hormones.

Previous Serial Number: NIAMD-LNE-4

Principal Investigator: Dr. Robert W. Bates

Other Investigators: None

Cooperating Units: None

Man Years

Total:	2
Professional:	2
Other:	

Project Description:

Objectives: To isolate protein hormones from the pituitary glands of man and animals, functional, transplantable mouse or rat pituitary tumors, and blood, especially the anterior pituitary hormones; to investigate the nature of these hormones as they circulate in the blood stream; to correlate chemical structure and biological function; to study the effect of hormones produced in excess by tumors on the host animal; to investigate the induction of diabetes by hormones.

Methods Employed: Hormones and other substances are extracted from pituitary gland, rat or mouse pituitary tumors, pancreas and blood. Crude extracts which may contain several hormones are fractionated by techniques commonly used in protein chemistry, such as salt precipitation, solvent fractionation, iso-electric precipitation, preparative and disc electrophoresis, ion-exchange chromatography, and gel filtration. Various quantitative biological assays are required to determine the concentration of the several hormones in tissues and protein fractions. Radioimmunoassay techniques are employed to extend the studies to concentration levels beyond the reach of conventional bioassay procedures. Removal of various endocrine glands with and without hormonal replacement is a critical technique. Effects of elimination or modification of hormonal production of the thyroid by anti-thyroid drugs and of the adrenal cortex by anti-adrenal drugs is compared to effects of surgical removal of the gland.

Major Findings: Hormonal Induction of Diabetes with Exogenous Hormones: Most of our studies this year have been concerned with the hormonal induction of diabetes (glucosuria) in rats with intact pancreas when the rats were on a diet containing 0.5% tolbutamide. Rats were injected twice daily for 5 days with bovine growth hormone (BGH), ACTH, dexamethasone, cortisol or one of the six paired-combinations of the four hormones. Glucosuria was measured quantitatively daily and when the rats were killed on the morning of the fifth day, 16 to 20 hours after the last injection, samples of blood and pancreas were taken for determination of their insulin concentration.

These experiments with tolbutamide in the diet served to confirm the synergistic interaction of the four hormones reported with rats on the regular diet. The greatest synergisms were between BGH and dexamethasone and between BGH and cortisol, with almost no synergism between BGH and ACTH as diabetogenic agents.

The time course of development was such that when dosages were two to four times the minimum effective dose, glucosuria did not develop until the third or fourth day with the regular diet whereas it was present already by the second or third day with tolbutamide in the diet. With the tolbutamide diet the dose-response curves with single hormones or pairs of hormones were similar to those previously reported for rats on the regular diet except that the hormonal dosage required to induce diabetes was reduced to about one half. These data suggest that tolbutamide is diabetogenic.

On day 5, if the rats had glucosuria, the blood sugar would be 200 to 300 mg% depending on the severity of the glucosuria. Hence the blood sugar levels tended to increase with dosage. With tolbutamide in the diet the blood sugar levels were too variable to be different from those on the regular diet. Similarly the level of insulin in the blood serum on day 5 was elevated 3 to 5 fold in glucosuric rats and this plateau level was independent of tolbutamide in the diet.

The concentration of insulin in the pancreas, however, was regularly lowered more at a given dosage level of hormones when tolbutamide was in the diet. That tolbutamide is diabetogenic is suggested by the earlier onset of glucosuria and the obvious decrease in insulin stores in the pancreas.

One side reaction observed was the formation of gastric ulcers with the higher dosages of the glucocorticoids, especially dexamethasone. This confirms the work of others. Ulcer formation was associated with a decrease or even cessation of food intake which often greatly reduced glucosuria by the 5th day and thus interfered with the progressively greater glucosuria each day observed previously in partially pancreatectomized rats, which required only one fifth the hormonal dosage and did not develop gastric ulcers.

Time course studies suggest that the hormones have little effect on the parameters studied during the first 4-6 hours. The insulin level in the blood then rises more rapidly than the glucose level so there is a fall in glucose/insulin ratio. After repeated injections this ratio then rises

back to a normal range even though glucose levels are elevated to 200-300 mg%. Decrease in this ratio may be one of the most important signs of prediabetes. At this later time the level of insulin in the pancreas is decreased but so far we have been unable to exhaust the insulin producing capacity of the intact pancreas so that permanent diabetes occurs. This must take a longer time than 5 days or larger doses of hormones, which would not be practical due to ulcer formation.

Significance to Bio-medical Research and the Program of the Institute: One of the major roles of the six hormones of the anterior pituitary gland is to modify or regulate the size and functional activity of other glands or tissues, which in turn control the overall metabolic state of the individual. This hormonal balance or imbalance controls how one feels and what one can do. Certain metabolic abnormalities and tumors are corrected by treatment with hormones or by removal of endocrine glands. Purified hormone preparations are needed for this work together with sensitive and quantitative tests which will estimate the concentration of hormones in the blood or transport system and the rate of release of the hormones. One needs to know what factors control the biosynthesis and subsequent release of hormones by the pituitary gland.

The studies on induction of diabetes reveal the major role of interaction in a synergistic manner of at least three hormones or types of hormones. The extent of this synergism is such that hormonal induction of diabetes in man should be seriously investigated. The use of tolbutamide as an antidiabetic agent is contraindicated by our studies so far.

Proposed Course: Studies on the induction of diabetes by hormones in rats that are 80% pancreatectomized will be carried out in a systematic manner to throw light on the problem of hormonal interaction (potentiation or inhibition) and to assess the significance of hormonal balance.

Analysis of hormone levels in rats with transplantable pituitary tumors will continue. A search for factors which modify these levels will be made. The physiological action of the hormones from the pituitary tumors upon the host will be studied. Studies on the changes occurring after tumor removal are planned.

The question of identity of human GH and prolactin results in confusion when one attempts to establish potency of prolactin in plasma of humans in various clinical conditions. Also the specificity of the cropsac response in pigeons raises the question of whether the response obtained with urine and plasma extracts is due to prolactin. These problems are being investigated.

Honors and Awards: None

Publications:

Bates, Robert W., Hormonal Induction of Diabetes. In: Diabetes, Rodriguez, R. R. (Editor) Excerpta Medica, Amsterdam, 1971. p. 757-770.

Bates, Robert W. and Garrison, Mary M., Studies in 80% pancreatectomized rats of the synergistic interaction of growth hormone, ACTH and the glucocorticoids (corticosterone, cortisol, prednisone and dexamethasone) as diabetogenic agents. Endocrinology 88, 1429-1436, 1971.

Serial No. NIAMD-LNE-5
1. Nutrition and Endocrinology
2. Endocrinology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Diabetes and Fat Metabolism

Previous Serial Number: NIAMD-LNE-5

Principal Investigators: Drs. Robert O. Scow and Sidney S. Chernick

Other Investigators: Dr. Margit Hamosh, Visiting Scientist
Dr. Carole Mendelson, Postdoctoral Fellow
Dr. Oren Zinder, Guest Worker
Dr. Cecilio Linder, Guest Worker
Dr. Vanco Kovacev, Skopje, Yugoslavia

Cooperating Units: NIAMD-LNE-6

PL-480 Agreement No. 02-009-1 with the
Department of Physiology, University of Skopje
Skopje, Yugoslavia

Man Years:

Total:	6-1/2
Professional:	3-1/2
Other:	3

Project Description:

Objectives: To determine influence of hormones and other factors on metabolism of fat and carbohydrate in liver, adipose tissue and mammary gland of normal and diabetic animals.

Methods Employed: Animals are deprived of one or several endocrine glands and treated with hormones or other substances. The effect of the above on the metabolism of fat and carbohydrate in liver, adipose tissue and other organs and on blood hormone levels are determined with in vivo and in vitro studies using conventional and radioisotope techniques, and electron microscopy. Effects of hormones added in vitro are also studied.

Major Findings: Metabolism of chylomicrons and lipoprotein lipase activity in perfused adipose tissue: Earlier studies in perfused rat adipose tissue showed that during uptake of chylomicron-triglyceride, triglyceride was hydrolyzed within the capillary endothelium to FFA and glycerol, and that two-thirds of the FFA were incorporated into tissue glyceride and the rest were released to the blood stream. More recent studies showed that the molar

ratio of FFA to glycerol released to the blood was 5.1 during the first 30 seconds of infusion of chylomicrons and less than 1.4 during the rest of the infusion. These findings suggest that partial glycerides are formed during the first stage of uptake of blood triglyceride by adipose tissue. The following hypothesis for transport of triglyceride-fatty acids across the capillary endothelium, based on our recent biochemical and cytochemical studies, is now being tested. It is proposed that soon after a chylomicron becomes attached to the luminal surface of the capillary endothelium, triglyceride in the chylomicron core is hydrolyzed by lipoprotein lipase to diglyceride and FFA, and the diglyceride becomes attached to enzyme bound to the endothelial plasma membrane, and the FFA is released to the blood stream. Vacuoles and microvesicles enclosing the diglyceride-enzyme complex are then formed and as they cross to the basal surface of the endothelium, the diglyceride is hydrolyzed to monoglyceride and FFA. The contents of the vacuoles and microvesicles are then discharged to the basement membrane where the monoglyceride is hydrolyzed to FFA and glycerol; the FFA diffuse to fat cells and the glycerol diffuses to the blood stream.

Metabolism of chylomicron-triglyceride in perfused rat mammary gland: A technique has been developed for perfusing the inguinal-abdominal mammary tissue of lactating rats. This preparation is being used to study the role of lipoprotein lipase in the uptake of chylomicron-triglyceride by mammary tissue. Perfused tissue of suckling rats retained in tissue lipid 10% of the chylomicron triglyceride-fatty acids infused and released 7% as FFA to the blood stream. The tissue also retained 9% of the chylomicron triglyceride-glycerol infused and released 6% as free glycerol. The molar ratio of FFA to glycerol released to blood was 15 during the first few minutes of chylomicron infusion, suggesting that partial glycerides were formed during the early stages of uptake. Non-suckling for 18 hours, which completely suppresses lipoprotein lipase activity in lactating mammary tissue, reduced by more than 80% both retention and hydrolysis of chylomicron-triglyceride. The findings show that hydrolysis occurs during the uptake of blood triglyceride by mammary tissue and that the rate of uptake is related to the level of activity of lipoprotein lipase in the tissue.

Hormonal control of lipoprotein lipase activity in mammary and adipose tissue during lactation: Previous studies in this laboratory showed that lipoprotein lipase activity is increased in mammary tissue and decreased in adipose tissue during lactation, and that when suckling is stopped, lipolytic activity decreases in mammary tissue and increases in adipose tissue. Recent studies in hypophysectomized lactating rats, given various combinations of the hormones needed for maintenance of lactation, showed that prolactin alone increases, within 24 hr, lipoprotein lipase activity in mammary tissue and suppresses the activity in adipose tissue; the effects of prolactin were enhanced slightly by concurrent injection of dexamethasone or of dexamethasone with growth hormone and thyroxine. Prolactin has no effect on lipoprotein lipase activity in nulliparous animals.

Lingual lipase: A very potent lipolytic activity was recently discovered in rat tongue during a search for the source of lipolytic activity found in the stomach contents of suckling rats. Further studies have shown that the activity is present mainly in lingual serous glands, located in the posterior part of the tongue, and that the activity is secreted into the oral cavity through ducts which open into the furrows of the circumvallate papilla and the foliate papillae. The enzyme activity in the tongue of both suckling and adult rats hydrolyzes triglyceride to diglyceride, monoglyceride, and FFA; about 2/3 of the glycerides formed are diglyceride and the rest are monoglyceride. The pH optimum of the reaction is 4.5-5.5. The enzyme activity is also found in saliva and gastric contents of rats fed a mixture of bovine milk and cream. The activity in gastric contents is negligible, however, when saliva is excluded from the stomach. Studies in suckling and adult rats showed that FFA account for about 1/3 of the fatty acids present in the stomach after a fat meal and that the pH of gastric contents during digestion is 4.5-5.5.

These findings suggest that the serous glands of tongue in the rat secrete a potent enzyme, hereon called lingual lipase, which hydrolyzes triglyceride in stomach contents to partial glycerides and FFA.

Significance to Biomedical Research in the Program of the Institute: Uptake of blood triglycerides by most tissues involves hydrolysis of triglyceride to FFA and glyceride by lipoprotein lipase. Cytochemical studies reported last year showed that hydrolysis in adipose tissue occurs in the capillary endothelium and basement membrane area. The studies in perfused adipose tissue described above suggest that hydrolysis of triglyceride occurs stepwise, with partial glycerides formed at the luminal surface, and hydrolysis completed in the basement membrane area. The studies in perfused mammary tissue also show that partial glycerides are formed during the early stages of uptake of blood triglyceride.

It is possible that lipoprotein lipase hydrolyzes triglyceride only to monoglyceride and that another enzyme, in the capillary wall, hydrolyzes monoglyceride to glycerol. If uptake of triglyceride should involve two different enzymes, glyceride could accumulate in the capillary endothelium if lipoprotein lipase was more active than the monoglyceride lipase. Whether such a process could occur in capillaries, or in the endothelium of larger vessels, should be studied.

Previous studies showed that lipoprotein lipase activity increases in mammary tissue and decreases in adipose tissue during lactation, and that this serves to direct dietary fatty acids to mammary tissue for the formation of milk lipid. It was also shown that nonsuckling for several hours decreases lipoprotein lipase activity in mammary tissue and increases that in adipose tissue. Since suckling also stimulates prolactin secretion, the findings in hypophysectomized lactating rats suggest that the effects of suckling on lipoprotein lipase activity in mammary and adipose tissue are mediated through the secretion of prolactin by the pituitary.

Last year it was reported that a potent lipolytic activity had been discovered in rat tongue which hydrolyzes triglyceride to partial glyceride (mostly diglyceride) and FFA at the same pH, 4.5-5.5, as that in the stomach following a meal. Recent studies showed that this lipolytic activity is present in saliva and that it is necessary for the hydrolysis of triglyceride in the stomach. Studies in several species, including man, have shown that 20-30% of the fatty acids in the stomach after a fat meal are FFA. Since partial hydrolysis of 60-80% of the triglyceride in the stomach would account for this amount of FFA, these findings are compatible with the concept that partial hydrolysis in the stomach also occurs in other species. It is proposed that the serous glands of the tongue secrete an enzyme, hereon called lingual lipase, which mixes with food in the mouth and hydrolyzes in the stomach the dietary triglyceride to diglyceride and FFA. It is suggested that conversion of the lipid to partial glycerides and FFA in the stomach facilitates emulsification of the lipid before it enters the duodenum.

Proposed Course: Adipose tissue will be perfused with emulsions of triglyceride containing labeled fatty acids in specific positions in order to determine more precisely where and how the triglyceride molecule is hydrolyzed after it is removed from the blood. Emulsions of partial glycerides will also be perfused to determine if they can be utilized by the tissue, and if so electron microscope cytochemical studies will be made to localize where in the capillary wall they are hydrolyzed.

Perfusion studies of lactating mammary tissue will be continued to study in what form glyceride-fatty acids enter the mammary epithelial cells. The large retention of chylomicron glyceride-glycerol in the tissues suggests that either partial glycerides or glycerol liberated from chylomicrons are utilized by the tissue. Incorporation of chylomicron fatty acids into milk lipid will also be studied.

Lingual lipase activity has been studied mostly in rats. Studies will be made to determine if the enzyme is present in other species, including man. Studies will also be made to determine its role in lipid digestion and to determine what factors regulate its synthesis and secretion. Isolation and purification of the enzyme will be initiated as soon as an ample source can be found.

Honors and Awards: None

Publications:

Scow, R.O. and Chernick, S.S. Action of pituitary and adrenal hormones in the development of diabetic ketosis. In: Rodriguez, R.R. and Vallance-Owen, J. (Ed.) Diabetes, Amsterdam, Excerpta Medica, Int. Congr. Ser. No. 231, 1971, pp. 771-780.

Chernick, S.S., Clark, C.M., Gardiner, R.J. and Scow, R.O. Role of lipolytic and glucocorticoid hormones in the development of diabetic ketosis. Diabetes In Press.

Serial No. NIAMD-LNE-6
1. Nutrition and Endocrinology
2. Endocrinology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Transport of lipid, hormones and enzymes through cells and membranes

Previous Serial Number: Extension of Project Serial No. NIAMD-LNE-5

Principal Investigators: Drs. E. Joan Blanchette-Mackie and Robert O. Scow

Other Investigators: Peter Berendsen, Guest Worker

Cooperating Unit: None

Man Years:

Total:	2-1/2
Professional:	1-1/2
Other:	1

Project Description:

Objectives: To study the structure and metabolism of cells that synthesize and secrete plasma lipid and hormones. To study the transport of lipid, hormones and enzymes across the capillary endothelium, basement membrane and extracellular space in different kinds of tissues. To study the uptake of lipid, hormones and enzymes by different kinds of cells. To correlate structural and biochemical changes induced in cells and tissues by various means.

Methods Employed: Animals of different ages and in various physiological states (fasting, pregnant, lactating, diabetic) are used to study with biochemical and morphological techniques the synthesis and secretion of lipoprotein triglyceride by liver and intestines, and the utilization of plasma triglyceride by adipose tissue, mammary gland and muscle. Perfused adipose and mammary tissues are used to localize with cytochemical techniques the sites of action of lipoprotein lipase and the mode of transport of fatty acids between blood and tissue cells. Perfused tissues are also used to determine with immuno-cytochemical techniques the distribution and site of synthesis of lipoprotein lipase and the transport of hormones across capillary endothelium and extracellular space. Isolated chylomicrons and purified lipoprotein lipase incubated in vitro are used to study the morphological changes in chylomicrons induced when the triglyceride core is reduced by lipolysis.

Major Findings: Role of the capillary endothelium in the uptake of chylomicron-triglyceride: The sites of hydrolysis of chylomicron-triglyceride and the mode of transport of glyceride fatty acids across the capillary wall have been determined in perfused adipose tissue with an electron microscope cytochemical procedure recently developed in this laboratory. The findings showed that plasma glyceride is hydrolyzed by lipoprotein lipase in the capillary endothelial cells and subendothelial space (basement membrane area), but not in the capillary lumen, or in or near fat cells. The results also indicated that glyceride-fatty acids are transported across the endothelial cells within a membrane-bounded system.

Cytochemical studies in adipose tissue perfused with both chylomicrons and insulin showed a marked increase in cytochemical reaction within the endothelial cell. This finding suggests that insulin increases uptake of glycerides by the endothelium. Whether insulin affects the removal of glyceride from the capillary endothelium is not known.

Infusion of heparin caused the immediate release of lipoprotein lipase activity to the blood stream and decreased the uptake of triglyceride by perfused adipose tissue. Five to ten minutes after stopping the infusion of heparin, cytochemical studies showed that lipoprotein lipase activity was present in the endothelium and in the subendothelial space, and biochemical studies showed that uptake of triglyceride was partially restored to normal.

The origin of lipoprotein lipase activity in the endothelial cells of adipose tissue has not been determined. Several studies have shown that isolated fat cells contain and synthesize the enzyme. The pronounced cytochemical reaction for lipase found in the subendothelial space near pericytes suggests that these cells may also produce or control lipoprotein lipase. Additional studies are needed to determine where the enzyme is synthesized and how its activity is regulated.

Uptake of plasma triglyceride by aortic endothelium: Cytochemical studies in the aorta of rats 5 min after intravenous injection of chylomicrons showed that plasma glycerides are hydrolyzed at the luminal surface and within membrane bounded vesicles of the endothelium. Glyceride was also found in the tunica intima and media of the aorta.

Metabolism of chylomicrons in vitro: Chylomicrons are spheres of triglyceride, with traces of cholesterol ester, enveloped by a surface coat of phospholipid, cholesterol and protein. When chylomicrons are incubated with lipoprotein lipase in postheparin plasma, the triglyceride is hydrolyzed to FFA and glycerol, and the latter are released to the incubation medium. Studies using the transmission and scanning electron microscopes showed that the surface coat of chylomicrons collapses inward and becomes wrinkled when the triglyceride content of the core is decreased by the action of lipoprotein lipase. The findings indicate that the surface coat is pliable and resistant to the action of the enzyme. The depleted chylomicrons have a deeply pitted surface, containing the initial phospholipid, protein and cholesterol and a

reduced core, with less triglyceride and the initial cholesterol ester. They are probably similar to the chylomicron remnants, observed in vivo by Redgrave. It is currently thought that the chylomicron remnants transport the residual lipid and protein to the liver.

Uptake of lipid and secretion of lipoproteins by intestinal epithelial cells of suckling rats: Electron microscopic examination of duodenal epithelium of suckling rats showed lipid droplets in membrane bounded cisternae of the duodenal epithelial cells, and chylomicrons in interepithelial spaces and lacteals. There was no evidence, however, of uptake of particulate lipid from the duodenal lumen by the epithelial cells. These results are compatible with the concept that FFA and monoglyceride are taken up by the epithelial cell and incorporated into particulate triglyceride within intracellular cisternae.

Particles similar in appearance and size to very low density lipoprotein were in the membrane bounded cisternae of duodenal epithelium and in the interepithelial spaces in unsuckled but not in suckled one-day old rats.

Significance to Biomedical Research in the Program of the Institute: Insulin treatment over a prolonged period has been linked to the development of vascular diseases in diabetics. The cytochemical studies described above showed that the capillary endothelial cell plays a major role in the uptake and transport of plasma lipids from the blood to parenchymal cells. The studies also showed that insulin causes accumulation of glyceride in capillary endothelial cells of perfused adipose tissue. It is possible that insulin may have a similar effect in endothelial cells of larger blood vessels and that this effect could be related to the deposition of excess lipid in arteries of long term treated diabetics.

Alterations in the metabolism of plasma triglycerides occur in hyperlipemic states. In some, synthesis and secretion of lipoproteins are abnormal whereas in others uptake is reduced, with and without changes in lipoprotein lipase activity. Localization of lipoprotein lipase synthesis and activity in tissues and study of the nature of the complex of lipoprotein lipase with chylomicrons and other lipoproteins could provide valuable information concerning factors that regulate or affect clearance of triglyceride from blood.

Proposed Course: Our cytochemical findings show that hydrolysis of chylomicron-triglyceride by lipoprotein lipase occurs within the capillary endothelium of adipose tissue. Although fat cells are thought to be the main source of this enzyme the manner in which the enzyme could be transferred to the endothelium has not been determined. An attempt will be made to develop an immuno-cytochemical technique for localizing lipoprotein lipase. With such a technique, one could determine whether the enzyme is present in parenchymal cells, pericytes, or in connective tissue components of the tissue. The effect of hormones and other factors on transport of the enzyme from sites of synthesis to sites of action could also be studied.

Sites of lipoprotein lipase synthesis and activity will be studied also in perfused lactating rat mammary tissue, skeletal muscle, and heart. Uptake and hydrolysis of triglyceride by arteries in different tissues and the effect of diabetes and insulin on these processes will be studied.

The nature of the enzyme-substrate complex formed between lipoprotein lipase and chylomicrons will be studied *in vitro* and *in vivo*. It is believed that the small amount of protein present on the chylomicron surface is necessary for the formation of the complex. Chylomicrons reacted with purified lipoprotein lipase will be studied structurally to determine if the enzyme has a direct effect on the chylomicron surface coat. The nature of the interaction *in vivo* between chylomicrons and lipoprotein lipase, presumably located on the luminal surface of capillary endothelial cells, will be studied, using a combination of immuno-cytochemical, freeze-etch and other techniques.

Hypertriglyceridemia and fatty liver occur in uncontrolled diabetes and in late pregnancy. The circulating lipoproteins in these states will be isolated and studied with the electron microscope to compare their structure with that in control animals. Since intestinal epithelial cells and hepatic parenchymal cells both synthesize plasma lipoproteins, and liver cells can store triglyceride, various components of these cells will be isolated for studies of nascent lipoproteins and cellular lipids in control, pregnant and diabetic animals.

Honors and Awards: None

Publications:

Blanchette-Mackie, E. Joan and Scow, Robert O. Sites of lipoprotein lipase activity in adipose tissue perfused with chylomicrons: Electron Microscope Cytochemical Study. *J. Cell Biol.* 51, 1 (1971).

Serial No. NIAMD-LNE-7
1. Nutrition and Endocrinology
2. Membrane Regulation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Mechanism of Action of Hormones on Adenylate Cyclase Systems

Previous Serial Number: NIAMD-LNE-6

Principal Investigator: Dr. Martin Rodbell

Other Investigators: Dr. Michael C. Lin, Visiting Scientist
Dr. Yoram Salomon, Visiting Fellow
Dr. James P. Harwood, Guest Worker
Dr. Boanerges Rubalcava, Guest Worker
Dr. Constantine D. Londos, Guest Worker
Ms. Vivian Goldberg, Predoctoral Fellow

Cooperating Units: Dr. Victor Hruby, University of Arizona

Man Years

Total	4
Professional:	3
Other:	1

Project Descriptions:

Objectives: To determine the mechanism of action of various hormones (peptide, catecholamines, prostaglandins) on adenylate cyclase systems in membranes from various animal cells.

Methods Employed: Cell membranes from a variety of cell types (human blood platelets, liver, adrenal cortex, erythrocytes, and heart) are purified by differential centrifugation on discontinuous gradients of sucrose. Adenylate cyclase activity and binding of labeled hormones were carried out by procedures developed in this laboratory.

Major Findings:

Regulation of Adenylate Cyclase Systems: Adenylate cyclase systems in animal cells are localized in the plasma membrane and are stimulated by a variety of peptide hormones, biogenic amines and prostaglandins. These systems are composed of a regulatory component (or receptor) and adenylate cyclase, the enzyme that converts ATP to cyclic AMP. The hepatic enzyme system of the rat responds to glucagon which binds to the regulatory component through reversible, non-covalent forces. In this system, the regulatory component is linked to the enzyme in a manner such that binding of

glucagon results in rapidly reversible changes in the activity of the enzyme when substrate (ATP) is provided at concentrations higher than 10^{-4} M. At lower substrate concentrations no hormone effect is observed unless either GTP or GDP (10^{-8} to 10^{-6} M) is present. Since the guanyl nucleotides are not substrates for the enzyme, it is assumed that they act at some site in the system other than the catalytic site on the enzyme. Similar actions of GTP have been observed on the glucagon-sensitive system in the beta cell of the pancreas and on the prostaglandin-sensitive system in human platelets.

The exact site of action of the nucleotides remains unknown but their actions on the hepatic system result in an increased rate of dissociation of glucagon from the regulatory component without changing the rate of association of the hormone to this component. This change in the affinity of the regulatory component for glucagon is considered to reflect an alteration in the "coupling" of the regulatory and catalytic components due to nucleotide binding at a regulatory site on the catalytic component.

Phospholipids appear to be involved in the binding of glucagon, coupling of regulatory and catalytic components, and in the state of activity of the enzyme. Treatment with detergents and phospholipase A results in loss of both binding and action of glucagon, but an increase in the response of the catalytic component to fluoride ion which enhances the activity of all adenylate cyclase systems even when the regulatory components have been removed by proteolytic digestion or by solubilization of the enzyme. Phospholipase C from B. cereus, which catalyses the hydrolysis of all phospholipids in liver membranes except sphingomyelin, causes a loss of response of the hepatic system to glucagon without loss of the fluoride response. In contrast, phospholipase C from Cl. perfringens, which catalyses the hydrolysis of sphingomyelin, lecithin, phosphatidylethanolamine, but not acidic phospholipids (phosphatidylserine or phosphatidylinositol), only slightly reduces the response to glucagon or fluoride ion even though the percentage of phospholipids hydrolyzed in the hepatic membrane by both phospholipases is the same. Such findings suggest that acidic phospholipids, present in only small amounts in the membrane, may be specifically involved in the processes through which glucagon stimulates the enzyme system.

In contrast to the hepatic system, the adenylate cyclase system in fat cells of the rat consists of several regulatory components each specific for epinephrine, ACTH, secretin, glucagon, LH, and TSH. These components are linked to a single catalytic component. As with the hepatic system, treatment with detergents and phospholipase A results in loss of all hormone responses but in retention or enhancement of the fluoride response.

The fat cell system responds fully to epinephrine in the presence of very low concentrations of ATP or AMP-PNP (a substrate for adenylate cyclase that is not hydrolyzed at its terminal phosphate). Addition of GTP results in marked inhibition of epinephrine stimulation and of basal activity. This effect is not observed with GDP and is dependent upon maintenance of the concentration of GTP. Activation of the enzyme by fluoride ion is also inhibited by GTP. It is thought that the inhibitory action of GTP is

through phosphorylation of the enzyme which not only reduces enzyme activity but also reduces the interaction of the epinephrine receptor with the enzyme and the ability of fluoride ion to activate the enzyme.

Concentrations of GDP as low as 10^{-7} M are required for the action of ACTH on fat cell adenylate cyclase when the concentration of ATP is reduced to 0.1 mM. ATP, at concentrations above 0.5 mM, mimicks the action of GDP. The same obligatory requirement for GDP at low substrate concentrations was observed for the ACTH-stimulated cyclase system in the adrenal cortex. GDP also stimulates basal activity in the adrenal system, suggesting that the nucleotide acts primarily on the enzyme causing a change in conformation necessary for interaction of the ACTH receptor with the enzyme (the same postulate suggested for the role of guanyl nucleotides in the action of glucagon on hepatic and pancreatic systems).

The Role of Calcium Ion in ACTH Action: Calcium ion is specifically required for the actions of ACTH on lipolysis and steroidogenesis in fat and adrenal cortical cells, respectively. Removal of calcium ion by a calcium chelator results in loss of response of fat cell cyclase to ACTH without changing the response of the enzyme system to glucagon, secretin, and catecholamines. Addition of a calcium chelator also causes loss of response of the adrenal system to ACTH. However, in the presence of GDP, calcium chelation results in enhancement of adrenal cyclase activity to levels observed when ACTH is added in the presence of GDP and calcium ion. Thus, calcium ion appears to play a key role in the regulation of adrenal cyclase activity provided that the enzyme is in its nucleotide "charged" state. ACTH acting through its receptor and calcium chelation bring about the same state of activity of the enzyme suggesting that ACTH may cause the removal or shift of calcium ion from its regulatory site.

Significance to Bio-medical Research and the Program of the Institute: Adenylate cyclase is a key regulatory system for the initial actions of a number of peptide hormones, biogenic amines, and prostaglandins. It is, accordingly, fundamental for an understanding of the molecular basis of hormone action to have complete knowledge of the physico-chemical nature of this membrane-bound system. Our studies indicate that hormonal interaction with receptors, though an obligatory step in hormone action, is not sufficient to bring about stimulation of adenylate cyclase. Nucleotides (notably guanyl nucleotides) are required for hormone action on a number of adenylate cyclase systems irrespective of the nature of the hormone receptor. The ubiquitous actions of the nucleotides suggest that they act by binding to a regulatory site on the enzyme which influences the process of "coupling" of the receptor and the enzyme. Another consequence of this action, seen with the glucagon system, is a marked increase in the rate of dissociation of the hormone from its receptor. We believe the latter to be due to the cooperative interaction of receptor with enzyme which results in changes in the affinity both of the enzyme for its substrate (MgATP) and of the receptor for its hormone. Put in another context, the cell's "receptiveness" to a particular hormone is determined by the availability of guanyl nucleotides at the level of the catalytic component irrespective of the levels of

circulating hormone available to the cell. Cellular regulation of receptor function in the cell membrane may be as important for hormone action as is the regulation of hormone release from the endocrine cells and may help to explain how certain endocrine disorders are manifested in spite of large circulating levels of hormones.

Proposed Course: Continued efforts will be made to isolate and characterize the various components of adenylate cyclase systems in liver, adipose, and adrenal cells. Through such efforts it may be possible to investigate the site of action of guanyl nucleotides and the means by which calcium ions and phospholipids influence the activity and regulation of the systems by hormones. Studies are in progress to determine the mechanism by which GTP inhibits the activity and response of the fat cell system to epinephrine.

In a collaborative project with Dr. Victor Hruby at the University of Arizona, successful solid phase synthesis of secretin has been accomplished. Analogues of the hormone are being prepared and will be used to investigate the structure-function relationships of secretin's action on the adenylate cyclase in fat cells.

Honors and Awards: None

Publications:

Pohl, S. L., Krans, H. M. J., Kozyreff, V., Birnbaumer, L., and Rodbell, M. The glucagon-sensitive adenylate cyclase system in plasma membranes of rat liver. VI. Evidence for a role of membrane lipids. J. Biol. Chem. 246, 447 (1971).

Birnbaumer, L., Pohl, S. L., Rodbell, M., and Sundby, F. The glucagon-sensitive adenylate cyclase system in plasma membranes of rat liver. VII. Hormonal stimulation: reversibility and dependence on concentration of free hormone. J. Biol. Chem. 247, 2038 (1972).

Pohl, S. L., Krans, H. M. J., Birnbaumer, L., and Rodbell, M. Inactivation of glucagon by plasma membranes of rat liver. J. Biol. Chem. 247, 2295 (1972).

Rodbell, M., Birnbaumer, L., Pohl, S.L., and Krans, H. M. J. Regulation of glucagon action at its receptor. In: Margoulies, M. and Greenwood, F. C. (Editors) Structure-Activity Relationships of Protein and Polypeptide Hormones. Vol. I. Excerpta Medica (Amsterdam), 1971, p. 199-211.

Rodbell, M., Birnbaumer, L., and Pohl, S. L. Characteristics of glucagon action on the hepatic adenylate cyclase system. Biochem. J. 125, 58 (1971).

Rodbell, M. In vitro assays of adenylate cyclase. In: Diczfalusy, E. (Editor) In Vitro Methods in Reproductive Cell Biology. Acta Endocrinologica, Suppl. 153 (1971), p. 337.

Rodbell, M., Birnbaumer, L., Pohl, S. L., and Krans, H. M. J. Hormones, receptors, and the adenylate cyclase system. In: Rodbell, M. and Condliffe, P. (Editors) Colloquium on Cyclic AMP (Fogarty International Publication) (1971).

Serial No. NIAMD-LNE-8
1. Nutrition and Endocrinology
2. Nutritional Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Biochemical Studies Related to Nutrition

Previous Serial Number: NIAMD-LNE-7

Principal Investigators: Drs. J. G. Bieri, J. N. Williams, Jr., and H. G. Windmueller

Other Investigators: Dr. R. E. Poukka Evarts, Dr. I. R. Peake, S. L. Thorp and A. E. Spaeth

Cooperating Units: Dr. S. Eisenberg, MD, NHLI
Dr. Peter Herbert, MD, NHLI
Dr. R. Krauss, MD, NHLI
Dr. William T. London, DVM, NINDS

Man Years
Total: 8
Professional: 6
Other: 2

Project Description:

Objectives - To determine the nutritional, biochemical and physiological role of essential nutrients for experimental animals. To define the metabolic function of certain nutrients and to study nutritional effects on the tissue levels of various metabolites.

Methods Employed - Rats, hamsters and guinea pigs are fed specially prepared, highly purified diets that contain adequate amounts of each nutrient known to be required by the particular species. The effects of specific deficiencies and imbalances are assessed by measurement of physiological, chemical and enzymological changes in the animal, its tissues and excreta.

The relationship of nutrition to other scientific areas, such as neurology and pathology, is studied.

Major Findings

A. Metabolism and Function of Fat-Soluble Vitamins (J. G. Bieri, I. R. Peake, and R. Poukka Evarts)

Studies have continued on the comparative metabolism of α- and γ-tocopherols (in collaboration with Dr. Windmueller). When an emulsion

containing the two tocopherols was infused into the stomach of rats with a lymph cannula, both compounds appeared in the lymph unchanged and were distributed similarly among the lipoproteins (40% in chylomicrons and 40% in very low density lipoproteins). When lymph containing a 1:1 mixture of the two tocopherols was injected intravenously, by 0.5 hour there was a 1.6 fold enrichment of α-tocopherol over γ-tocopherol in the plasma. Also, there was a rapid shift of both compounds to the high density lipoprotein fraction. The distribution of both tocopherols in plasma lipoproteins paralleled the distribution of total lipid. Tissues lost γ-tocopherol faster than α-tocopherol so that by 24 hours the ratio was 1:2, respectively. (Dr. Peake)

In a study of vitamin E in normal rhesus monkeys, removal of the vitamin from the diet resulted in a rapid fall in plasma concentration until by three weeks it was one-half the normal level. This concentration persisted for four more weeks when supplementation began. Dietary d-α-tocopherol at 5 mg/kg prevented a further decline of the plasma level but 10 mg/kg was necessary to restore the concentration to the normal range. It was concluded that 5 mg/kg diet is probably the minimum requirement and 10 mg/kg is an adequate amount. (Drs. Bieri and Evarts; in collaboration with Dr. London, NINDS)

Analysis of typical breakfasts, lunches and dinners from the NIH cafeteria for their vitamin E content revealed that the daily intake of α-tocopherol may range from 4 mg for low fat diets to 12.5 mg for high fat diets. Other major tocopherols present, and their amounts relative to α-tocopherol, were δ-tocopherol (1X) and γ-tocopherol (3X). (Drs. Bieri and Evarts)

B. Synthesis and Metabolism of Plasma Lipids and Lipoproteins (H. G. Windmueller)

A variety of in vivo and in vitro rat preparations have been established in the laboratory and were used in several related investigations. Preparations include isolated perfused liver, isolated perfused intestine with lymph drainage, rats with mesenteric lymph fistula and re-entrant bile fistula, and a supradiaphragmatic rat preparation.

Intestinal fatty acid synthesis de novo was quantitated both in vivo and in vitro by measuring the incorporation of tritium, from ^3H_2O, into the tissue and lymph fatty acids. Compared to liver and adipose tissue, the rate of fatty acid synthesis by the gut is low, and newly-synthesized fatty acids do not contribute significantly to mesenteric lymph lipids, as has been postulated by others.

Synthesis of the various apoproteins of the plasma lipoproteins was investigated in isolated liver and gut perfused with [^{3}H]lysine. (Collaboration with Dr. P. Herbert, NHLI) Synthesis of all known apoproteins was demonstrated with liver. The pattern of [^{3}H]lysine incorporation was not that expected from the relative quantitative contribution of the various apoproteins, however. Isolated intestine synthesized all the major lipoprotein apoproteins with the exception of a group of low molecular weight peptides that are found in both

very low density (VLDL) and high density lipoproteins (HDL). Lipoproteins isolated from mesenteric lymph in vivo, however, contained these peptides. Taken together, these results show that: (1) gut, as well as liver, is an important site of lipoprotein apoprotein synthesis; (2) liver and gut differ in their capacity to synthesize the various apoproteins; (3) nascent lipoproteins synthesized by liver and gut are rapidly altered by combining with apoproteins contributed by other lipoproteins already in the circulation.

To determine if plasma HDL is metabolized as a unit, the fate of I.V. injected ^{125}I-labeled rat and human HDL (>95% of label on protein) was studied in the rat. (Collaboration with Dr. S. Eisenberg, NHLI) At 5-minute to 72-hour intervals after injection, the radioactivity of the plasma lipoprotein apoproteins was determined as well as the radioactivity of the rat tissues. Studies with both rat and human HDL led to the following conclusions: (1) the disappearance of all apoproteins from plasma was exponential, but the rates of disappearance for all apoproteins were not identical, indicating that HDL is not metabolized as a unit; (2) the low molecular weight apoproteins of HDL equilibrate rapidly with those of VLDL; (3) liver is a major site for HDL apoprotein catabolism; (4) human and rat HDL were similarly metabolized in the rat.

The goal of collaborative studies underway with Dr. R. Krauss (NHLI) is to determine the relative importance of liver and extra-hepatic tissues as the source of the lipolytic activity released into the plasma following the injection of heparin. Results indicate that, contrary to current belief, the liver is the source of most of the activity. There are important qualitative differences, however, in the enzymatic activity released by liver and that from other tissues, and the physiological consequences of this are being explored.

C. Biochemical Studies of Cytochromes (J. N. Williams, Jr.)

We have obtained from well-washed rat liver mitochondria a subpopulation of particles (SPP) having properties similar both to conventional mitochondria and microsomes. In various sucrose density gradient centrifugations the SPP bands sharply and distinctly from conventional mitochondria and microsomes. The SPP appear to resemble mitochondria more than microsomes, however, for the following reasons: (1) Typical mitochondrial enzymes (cytochrome oxidase, monoamine oxidase, succinate cytochrome c reductase) are present at the same concentrations as in conventional mitochondria per unit of protein. A typical microsomal enzyme (glucose-6-phosphatase), which also peaks with the SPP on sucrose density gradients, is present in considerably lower concentration than in microsomes. Another microsomal enzyme (NADPH-cytochrome c reductase), while demonstrable in the unseparated SPP fraction, does not peak with the SPP on sucrose density gradients. (2) The SPP exhibits the same respiratory control with ADP as conventional mitochondria. (3) The SPP possesses the same low ATPase and NADH oxidase activities as conventional mitochondria. Attributes (2) and (3) indicate that the SPP are not degraded conventional mitochondria or fragments of such mitochondria. The sharpness and duplicability of the bands on sucrose density gradients of conventional

mitochondria and the SPP also indicate a genuine difference in the two particles. When mitochondria and SPP are re-mixed and separated on sucrose density gradients, two bands appear with peaks at the same densities as for each individual type of particle. When incubated with exogenous cytochrome c much more of the cytochrome is taken up by the SPP than by conventional mitochondria per unit of protein. Thus, the SPP carries more negative charges per unit of protein than conventional mitochondria.

Lysosomes and peroxisomes are present in the SPP fraction and can be distinctly separated from each other, and from SPP, in sucrose density gradients. In contrast to the single peaks reported by others, we have found that the lysosomal and peroxisomal contaminants of the SPP fraction each exhibit biphasic peaks, indicating that lysosomes and peroxisomes of at least two densities are present in rat liver.

D. Significance to Bio-Medical Research and the Program of the Institute

A more complete understanding of the nutrition, biochemistry and metabolism of essential amino acids, proteins, vitamins, minerals and fatty acids in different living organisms can be expected to contribute still further to our knowledge of the roles of these essential nutrients in human beings. It is well established that the nutrition of man plays a role in the etiology of many degenerative and metabolic diseases, certain infectious and neurological diseases and some types of cancer. Basic studies in nutrition and biochemistry of nutrients may provide the means to prevent or cure some of these diseases.

E. Proposed Course of Project

Clarification of nutritional responses and explanation of interrelationships at the biochemical level remain the primary goals of the project.

F. Publications

LaRosa, J. C., Levy, R. I., Windmueller, H. G. and Fredrickson, D. S.: A comparison of the triglyceride lipase of liver, adipose tissue and post-heparin plasma. J. Lipid Res., in press.

Peake, I. R. and Bieri, J. G.: Alpha- and gamma-tocopherol in the rat: in vitro and in vivo tissue uptake and metabolism. J. Nutr. 101: 1615-1622, 1971.

Peake, I. R., Windmueller, H. G. and Bieri, J. G.: The absorption of lymphatic transport of α- and γ-tocopherol in the rat. Biochim. Biophys. Acta, in press.

Rogers, W. E., Jr., Bieri, J. G. and McDaniel, E. G.: Vitamin A deficiency in the germfree state. Fed. Proc. 30: 1773-1778, 1971.

Windmueller, H. G. and Spaeth, A. E.: Fat transport and lymph and plasma lipoprotein biosynthesis by isolated intestine. J. Lipid Res. 13: 92-105, 1972.

Serial Number NIAMD-LNE-9
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Structure and function of barnase and barstar, an extracellular ribonuclease (barnase) and its intracellular inhibitor (barstar) from *Bacillus amyloliquefaciens*.

Previous Serial Number: LNE-8

Principal Investigator: Robert W. Hartley

Other Investigators: NONE

Cooperating Units: Large-scale laboratory, Office of Chief, LNE, NIAMD
Robert W. Hartley (on leave without pay) Wellcome Fellow at Sub-division of Protein Chemistry, Medical Research Council, Laboratory of Molecular Biology, Cambridge, England, in collaboration with B. S. Hartley and J. Sperling. Also other units under D. M. Blow and R. C. Sheppard at the same laboratory.

Man Years

Total:	1.5
Professional:	1.0
Other:	0.5

Project Description:

Objectives: To further purify, characterize, and investigate this enzyme and its natural inhibitor by physical, chemical, and biological techniques. To develop methods of culture, genetic manipulation and genetic analysis so that the organism and its product enzyme-inhibitor pair may be applied to a broad study of enzyme structure, function and synthesis.

Methods Employed: *B. amyloliquefaciens* (strain H and derivatives and several other strains all of which were formerly considered to be strains of *B. subtilis*) are grown in synthetic media in a variety of batch and continuous flow fermentations. Isolation of barnase involves: 1) adsorption onto P-cellulose from acidified culture medium, followed by stepwise elution, 2) desalting by Sephadex chromatography, 3) gradient chromatography on CM-cellulose. The inhibitor (barstar) is obtained from the cells after acetone and aqueous acid extractions by extraction at pH 8.5. It is purified by acid precipitation, DEAE-cellulose, and Sephadex G-75 chromatography and by making use of its specific binding to purified enzyme.

Work on the determination of amino acid sequence involves amino acid analysis, enzymatic digestions, peptide separations, and peptide sequencing by the combined Edman-dansyl techniques. Amino acid analysis is by automated liquid chromatography using a JEOLCO analyzer. Dansyl amino acid derivatives are identified by thin layer chromatography on polyamide layers. Peptide separations are obtained by Sephadex and ion-exchange chromatography.

The reversible transition undergone by barnase and its derivatives under the influence of heat or denaturing agents is studied primarily by ultraviolet absorption, but also by optical rotatory dispersion, exclusion chromatography, and with the use of protelytic enzymes.

Derivatives, such as those prepared by partial exopeptidase digestion, are also compared to native barnase with respect to activity, reaction with inhibitor and with anti-enzyme antibody, chromatographic behavior, crystal growth, etc.

Equilibrium ultracentrifugation has been used to measure the molecular weights of the enzyme, the inhibitor, and their complex, and to determine the molecular weight homogeneity of each. Disc and equilibrium pH gradient electrophoresis have also been used to assess homogeneity and to demonstrate the reversibility of the complex formation.

Bacterial mutants are obtained by chemical mutagenesis of vegetative cells, and by gamma irradiation of spores. A variety of techniques are used to isolate ribonuclease, protease, amylase, nutritional and antibiotic resistant mutants.

Radioactive amino acids are incorporated into barnase and into nascent barnase peptides by their addition to appropriately prepared cells. Attempts to obtain and identify nascent barnase peptides involve proteolytic treatment of spheroplasts.

Crystals of barnase are grown in hanging drops (6 to 8 µl) suspended over suitable solvents. The three-dimensional structure of barnase is being determined by the methods of X-ray crystallography.

The Merrifield procedure is being used to synthesize a portion of the barnase sequence.

Major Findings: Crystals of barnase suitable for X-ray crystallography may be grown in 30-40% saturated ammonium sulphate solution with the addition of zinc ions and β-mercaptoethanol. Neither of these additions affect the basic crystallographic structure, but do strongly affect nucleation and the relative growth of different crystal faces. The effect of mercaptoethanol is probably due to sequestration of contaminating traces of heavy metal ions (e.g., Hg^{++}). Crystals so grown exhibit a high degree of order and very good stability in the X-ray beam. The molecules are packed as trimers into a trigonal lattice, giving nine molecules per unit cell. Additional symmetry within the three-molecule assymetric unit greatly improves the chances for a

rapid solution of the structure.

Barnase radioactively labeled at all residues and specifically labeled at the three phenylalanines has been obtained. Strong evidence has been found in the course of this work that barnase as initially synthesized contains one less positive (or one more negative) charge than the purified material.

Significance to Bio-Medical Research and the Program of the Institute: The small size, stability, easily measured enzyme activity and absence of disulfide bridges in barnase made it an excellent choice for a long-range study of the relation between the structure of an enzyme and its function. It now appears that the inhibitor is an even simpler protein and should be useful in a similar fashion. Together they offer a model system for the study of a protein-protein interaction.

The nature and extent of the thermal transition of barnase gives strong support to the concept that the three-dimensional structure of a protein may be completely determined by its amino acid sequence. The simplicity and all-or-none nature of the transition should make it useful for thermodynamic analysis of the factors involved in maintaining a folded protein structure.

The production of both of these proteins by the same organism is also of biological interest.

Proposed Course of Project: Current experiments using radioactive labels to investigate the site of barnase synthesis will continue. The nature of the apparent post-synthesis modification of barnase will also be investigated. X-ray structure work will continue.

On the return of the principal investigator to the NIAMD, priority will be given to the growth of crystals of the barnase-barstar complex, and to the sequencing and crystallization of barstar.

Further characterization and study of the three entities, barnase, barstar, and their complex, and various derivatives of each will be carried out by as many techniques as possible. Comparison studies by the same techniques will also be applied to the homologous proteins produced by related strains of *B. amyloliquefaciens*. It is hoped that similar studies can be applied to altered proteins produced by genetic variants of these strains.

HONORS AND AWARDS: None

Publications:

Hartley, R. W., and Rogerson, D. L. Jr.: Production and purification of the extra-cellular ribonuclease of *Bacillus amyloliquefaciens* (Barnase) and its intra-cellular inhibitor (Barstar). I. BARNASE. Preparative Biochemistry, (in press)

Hartley, R. W., and Rogerson, D. L. Jr.: Production and purification of the extra-cellular ribonuclease of *Bacillus amyloliquefaciens* (Barnase) and its intra-cellular inhibitor (Barstar). II. BARSTAR. Preparative Biochemistry (in press).

Hartley, R. W., and Barker, E. A.: Amino acid sequence of extracellular ribonuclease (Barnase) of *Bacillus amyloliquefaciens*. Nature New Biol. 235: 15-16, 1972.

Hartley, R. W.: Peptide analysis and preparation with an amino acid analyzer. Anal. Biochem. 46: 676-680, 1972.

Serial No. NIAMD-LNE-10
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on structure and function of nucleic acids.

Previous Serial Number: LNE-9

Principal Investigator: Dr. G. W. Rushizky

Other Investigators: Mr. J. E. Mozejko, Mr. D. L. Rogerson, Jr., Dr. H. A. Sober

Cooperating Units: Large-scale laboratory, LNE, NIAMD

Man Years

Total:	2.3
Professional:	2.3
Other:	0

Project Description:

Objectives: To study the enzymology and chemistry of nucleic acids and to develop new methods for the determination of nucleotide sequences in RNA and DNA.

Methods Employed: Various systems for 2-dimensional polyacrylamide gel electrophoresis were examined with a view toward a mapping procedure for large oligonucleotides from enzymatic digests of RNA or DNA.

Other experiments dealt with the fractionation by polyacrylamide gel electrophoresis (in one dimension at neutral pH) of various stages of enzymatic hydrolysis of viral RNA by several (ribo)nucleases. Among these, the RNase of *B. amyloliquefaciens*, of *U. sphaerogena*, and of micrococcal nuclease were especially useful.

Major Findings: A procedure was developed for the counting of ^{32}P-labeled RNA hydrolysates fractionated by polyacrylamide gel electrophoresis, which involved strip chart counting with a gas flow counter, rather than by slicing and counting in a scintillation counter. Because of difficulties encounterd in growing ^{32}P-labeled bacterial viruses in liquid culture, a procedure for doing this on agar plates was developed. Several suitable bacterial viruses were examined as sources of homogeneous high molecular weight DNA. Φx 174 was found to be much more difficult to grow, in large yields, than the rod-like *E. coli* virus fd. DNA prepared from the latter will be used as a substrate for hydrolysis by various DNases.

Two-dimensional polyacrylamide gel electrophoresis of enzymatic digests of MS2 RNA and fd DNA give reproducible patterns in 2-dimensions, as do f-met- and arginine tRNAs.

Micrococcal nuclease, *B. amyloliquefaciens* RNase and *U. sphaerogena* RNase digests of MS2 RNA, were found to produce well-defined large oligonucleotide fractions of chain length comparable to that of tRNA. A paper on the fractionation of trinculeotides from partial micrococcal nuclease digests of calf thymus DNA (see last annual report) was published.

Significance to Bio-Medical Research and the Program of the Institute: The methods developed should be useful for the preparation, isolation and characterization of large oligomers from RNA and DNA.

Strip chart counting of ^{32}P-labeled oligomer fractions in polyacrylamide gels is much more reproducible and less laborious than slicing and counting in scintillation counters. Growth of ^{32}P-labeled viruses on agar plates rather than in liquid culture simplifies the handling of radioactive materials and the growth of a larger number of labeled viruses. Two-dimensional polyacrylamide gel electrophoresis of partial enzymatic digests of MS2 RNA and fd DNA gives reproducible patterns, as do digests of f-met- and arginine tRNA.

Because of their different specificities, the RNases of *B. amyloliquefaciens* and *U. sphaerogena*, as well as micrococcal nuclease, should complement the use of pancreatic RNase and RNase T_1 for the determination of overlapping nucleotide sequences in, as well as for, the preparation and isolation of large oligomers from high-molecular weight RNAs.

Honors and Awards: NONE

Publications:

Rushizky, G. W., Mozejko, J. H., Woodford, P. C. and Sober, H. A.: Fractionation of trinucleotides from partial micrococcal nuclease digests of calf thymus DNA. Anal. Biochem. 46: 443-452, 1972.

Rushizky, G. W., and Mozejko, J. H.: Fractionation by column chromatography in aqueous methanol of partial *Ustilago spaherogena* nuclease digests of RNA. Anal. Biochem. 43: 535-543, 1971.

Serial No. NIAMD-LNE-11
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Protein:Nucleic acid interaction: Chromatin structure and restriction in eukaryotic cells.

Previous Serial Number: LNE-10

Principal Investigator: Dr. Robert T. Simpson

Other Investigators: Dr. Shoshana Levy and Dr. Herbert A. Sober

Cooperating Units: NONE

May Years

Total:	2.0
Professional:	1.0
Other:	1.0

Project Description:

Objectives: To study the structure and enzymatic bases for the restriction of translation of the major portion of genetic information in nucleated, mammalian cells; and to study the mechanisms which allow transcription of that portion of the genetic message which is appropriate to the function of the particular cell type.

Methods Employed: Using established methodology, nuclei are isolated from mammalian cells, and then chromatin, the diffuse interphase form of the chromosomes, is isolated from these nuclei. The physicochemical properties of the isolated chromatin are studied using standard biochemical techniques, such as selective chemical modification, circular dichroism, absorption spectrophotometry, ultracentrifugation, hydrogen-ion titration, and interaction with reporter compounds.

Major Findings: In continuing attempts to understand the topological relationships of DNA and proteins in chromatin, the definition of the binding of histones to DNA has been studied. For this purpose, the acetylation of chromatin with acetic anhydride under controlled conditions (previously described) has been employed. This reaction would be thought to modify only those lysyl residues of the histones which do not bind to nucleic acid in the native complex. Hence, isolation of the histones and determination of those lysyl residues in the primary protein sequence of these proteins which are not modified, should indicate these regions of each histone which bind to DNA. From this information, reasonable models are derived for the binding of protein

to DNA in chromatin.

Calf thymus chromatin was acetylated with labeled acetic anhydride, the histones isolated, and then digested with trypsin to produce peptides. Since acetyllysine is not a substrate for tryptic hydrolysis, longer peptides than those obtained in tryptic digestions of control histones would be produced, with the length depending on the number of sequential lysyl residues modified for any given protein. Separation of the peptide digest on G-50 Sephadex in the presence of 20% formic acid led to isolation of six groups of labeled peptides, and demonstrated that while most of the isotope was present in the larger peptides, there was little associated with the very small species. These six groups have then been fractionated by sequential gradient chromatography on a sulfonic acid resin and a quaternary ammonium ion resin (for the two smaller groups) and on CM-cellulose and DEAE-Sephadex (for the four larger groups). These procedures have led to the resolution of about 85-90 distinct peptides, of which some 11-12 are radioactively labeled. These latter ones should correspond to the nonbound regions of the five main histone fractions. Compositional and sequence studies on these peptides are currently underway to allow their localization in the known primary sequences of the various histones.

In a different vein, there has recently been some indication that chromatin might possess regions where the DNA has a single stranded character, and this has been an important portion of Crick's postulated model for chromosome structure. In attempts to ascertain the presence or absence, and amount, of single stranded DNA in chromatin using anti-single strand DNA antibody induced in rabbits, quantitative complement fixation tests were performed with native and heat denatured chromatin, as well as controls with single and double stranded DNA, both alone and in their complexes with histones. It was demonstrated that there is no antigenically recognizable single stranded DNA in native chromatin (limit of detection less than 0.1%). These findings preclude this portion of the Crick model, and suggest that recognition in eukaryotic systems must either involve local unwinding of the double helix, or recognition of base sequences while the DNA is fully helical.

Significance to Bio-Medical Research and the Program of the Institute:
It is hoped that the studies on acetylated chromatin histones will allow ascertainment of the means of binding of these basic proteins to nucleic acids. Having this information, we will then attempt to evaluate the structural interrelationships of proteins and nucleic acids in chromatin of different differentiated states of a single cell (e.g., avian erythrocytes at different developmental levels, or different stages in the cell cycle), and in the separable forms of chromatin which have recently been obtained (See NIAMD-LNE-15). Such information on the structure of chromatin per se. and on alterations in its structure which correlate with functional changes in the cell, is paramount to the understanding of gene regulation in the cells of higher organisms.

Honors and Awards: NONE

Publications:

Simpson, R. T.: Modification of chromatin with acetic anhydride. Biochemistry 10: 4466-4470, 1971.

Simpson, R. T.: Modification of chromatin by trypsin: The role of proteins in maintainance of DNA conformation. Biochemistry (in press).

Yaron, A., Otey, M. C., Sober, H. A., Katchalski, E., Ehrlich-Rogozinski, S., and Berger, A.: Lysine oligopeptides. Preparation by Ion-Exchange chromatography. Biopolymers 11: 607-621, 1972

Serial Number NIAMD-LNE-12
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The large-scale purification of ribonuclease T1 and T2 from crude Japanese Takadiastase powder.

Previous Serial Number: LNE-11

Principal Investigator: Dr. Pamela Roddy

Other Investigators: Dr. G. W. Rushizky and Dr. H. A. Sober

Cooperating Units: None

Man Years

Total:	1.0
Professional:	1.0
Other:	0

Project Description:

Objectives: To purify large amounts of ribonuclease T1 and T2 from crude Japanese Takadiastase powder for specific enzymatic hydrolysis of RNA.

Methods Employed: The Takadiastase ribonuclease T1 and T2 enzymes were purified by $AmSo_4$ and acetone precipitation, DEAE-cellulose, and CM-cellulose chromatography using phosphate and Na-acetate buffers. The enzyme activity was assayed at pH 4.5 and 7.5 using yeast RNA as a substrate to determine T1/T2 ratios.

Major Findings: 4.1×10^5 A_{260} units of ribonuclease T1 with a specific activity of 8.4 and 1.0×10^3 A_{260} units of ribonuclease T2 with a specific activity of 8.5 have been purified.

Significance to Bio-Medical Research and the Program of the Institute: A large amount of pure ribonuclease T1 and T2 will be useful for the preparation of oligonucleotide fragments. Large amounts of oligonucleotides are needed for the following studies planned or in progress:

1) Study of physical and chemical properties and crystallization and X-ray diffraction studies on the individual trinucleotide and complementary pairs of trinucleotides so that background information may be obtained for the work on large oligonucleotide chains,

2) As reagents for the preparation of affinity adsorbents which can be

used for such studies as localizing sequence specific nucleases,

3) Possible antigens for the production of sequence-specific antibodies,

4) As substrates for kinetic studies of nuclease action, and

5) Analysis of circular dichroism of ribonucleotide trimers.

Honors and Awards: NONE

Publications:

Rushizky, G. W., Mozejko, J. H., Woodford, P. C. and Sober, H. A.: Fractionation of trinucleotides from partial micrococcal nuclease digests of calf thymus DNA. Anal. Biochem. 46: 443-452, 1972.

Serial Number NIAMD-LNE-13
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Fractionation of chromatin and the study of nonhistone proteins.

Previous Serial Number: LNE-12

Principal Investigator: Dr. Shoshana Levy

Other Investigators: Dr. Robert T. Simpson and Dr. Herbert A. Sober

Man Years

Total:	1.3
Professional:	1.3
Other:	0

Project Description:

Objectives: Development of a general method for fractionation of chromatin, and the isolation and characterization of its acidic proteins. To study the possible role of nonhistone proteins in restriction of template activity of chromatin.

Methods Employed: Chromatin is isolated from cell nuclei by established methods, and fractionated by ultracentrifugation and ion-exchange chromatography. The fractions obtained are analyzed using methods such as spectrophotometry, electrophoresis, amino acid composition and immunochemical assays.

Major Findings: The methodology described for fractionation of chromatin into its constituent species, and partial subfractionation of the nonhistone proteins has been completed. The final step, fractionation of the nonhistone proteins on DEAE-cellulose in the presence of 3 M urea, has been markedly improved by the introduction of a complex salt gradient for the elution, resulting in total separation of the two major groups of nonhistones, and partial resolution of several components within these two major groups.

With these methods the fractionation of the nonhistone proteins was performed at different stages of differentiation in various cell types. Study of the mature and immature avian erythrocyte have led to the detection of only minor differences in nonhistone proteins between these two functional states of the cell, one actively synthesizing globin, and the other essentially inactive in transcription and protein synthesis.

Stimulation of guinea pig lymphocytes with phytohemagglutinin leads to induction of marked metabolic activity, and after a lag phase of about 24 hours,

the onset of DNA synthesis followed by cell division. This system is one of several models for the transition from the quiescent to the dividing state which have been investigated in many laboratories. In collaboration with Drs. Ronald Levy and S. Rosenberg (NCI), the early changes in synthesis of histones and nonhistones in PHS stimulated lymphocytes were studied in conjunction with the recently developed chromatin fractionation procedure. During the first hour after stimulation, there is only a very slight synthesis of histones, and this does not differ from that observed for the control unstimulated samples. In striking contrast, even at this early phase in the induction of mitotic activity, a marked enhancement of syntehsis of nonhistone proteins occurs consequent to the stimulation. The increased synthesis apparently involves all the protein groups detected by either charge- or size-based electrophoresis on polyacrylamide gels, or by chromatography on DEAE-cellulose, although the possibility of highly stimulated synthesis of a protein present in relatively small amount can not be excluded. The changes documented by these studies occur in as short a time period after stimulation as any previously recorded for this system, and far precede the increase in synthesis of histones, RNA or DNA. The possible causal relationship of the changes in nonhistone protein synthesis, and the level of its control, are currently under investigation.

A third system under investigation is that originally described by Shelton and Allfrey. Cortisol treatment of adrenalectomized rats led to preferential synthesis of a nonhistone protein of about 41,000 molecular weight. Preliminary studies have confirmed the greater degree of synthesis of the nonhistones as a class (vs. the histones) in treated animals. Attempts to isolate the protein observed by Shelton and Allfrey by our fractionation procedure are currently under way.

Significance to Bio-Medical Research and the Program of the Institute: There is some evidence that specific restriction of chromatin template activity might be due to the presence of specific nonhistone protein components. We have developed methodology for isolation and partial fractionation of these proteins on a preparative scale. Using this methodology, we now attempt to evaluate the role of these proteins directly in the case of three systems where varying degrees of differentiation change is occurring. Such knowledge of the mechanism of gene regulation is a prerequisite to the understanding of cellular development,differentiation, and dedifferentiation.

Honors and Awards: NONE

Publications:

Levy, Shoshana, Simpson, R. T., and Sober, H. A.: Fractionation of chromatin components. Biochemistry 11: 1547-1554, 1972.

Yaron, A., Fasman, G. D., Sober, H. A. and Berger, A.: The chainlength dependence of the conformation for oligomers of L-lysine in aqueous solutions: optical rotarory dispersion studies. Biopolymers 10: 1107-1120, 1971.

Serial No. NIAMD-LNE-14
1. Nutrition and Endocrinology
2. Developmental Biochemsitry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Properties of folic acid γ-glutamyl carboxypeptidase.

Previous Serial Number: NONE

Principal Investigator: Dr. Gerald R. Reeck

Other Investigators: Mr. Howard Bakerman

Cooperating Units: None

Man Years

Total:	1.3
Professional:	1.3
Other:	0

Project Description:

Objectives: To purify folic acid γ-glutamyl carboxypeptidase (conjugase) and determine its physico-chemical properties and to study the action of the purified enzyme on polyglutamyl derivatives of folic acid, in particular that isolated from yeast.

Methods Employed: Protein isolation is pursued utilizing current methodology including ion exchange chromatography, gel filtration, and affinity chromatography. Enzymatic activity is assayed by microbiological techniques and thin-layer chromatography of reaction mixtures. Proteins are characterized by disc gel electrophoresis, ultracentrifugation, amino acid analysis and related techniques. The enzymatic properties of conjugase are studied by observing the effect of the enzyme on various polyglutamyl derivatives of folic acid, especially that from yeast.

Major Findings: Because of its high level of conjugase activity and its relatively cheap and ready supply, we have used chicken pancreas, either fresh or an acetone powder, as our starting material.

Our first goal was to obtain a stable preparation since previous experience in this laboratory had indicated the enzyme to be unstable after initial fractionation. This was accomplished by removal of trypsin, chymotrypsin or their zymogens either by affinity chromatography or gel filtration. Having obtained a stable preparation and characterized the behavior of the enzyme by ammonium sulfate precipitation, DEAE-cellulose chromatography, gel filtration and several potential affinity chromatographic systems, final purification is underway.

To facilitate this task we have developed a rapid assay to supplement the time consuming microbiological technique which has been utilized in this and other laboratories for years. The substrate used in our assay is a yeast folic acid derivative thought to have seven glutamyl residues, most of which are hydrolyzed during conjugase action to give a product having one or two glutamyl residues. The concomitant reduction in negative charge should allow separation by ion exchange chromatography of the heptaglutamate and the product of conjugase digestion.

The very sensitive and rapid technique of DEAE cellulose thin-layer chromatography is used for the analysis of conjugase reaction mixtures. We obtained good separation of starting material and product, eliminating the requisite overnight incubation and considerable manipulation of the microbiological assay and replacing these with a 20-minute chromatography followed by rapid analysis of the results. In addition to its utility in assaying for conjugase, the thin-layer system in combination with a variety of detection techniques at our disposal will provide a valuable tool in assessing the state of oxidation, ring substitution and glutamic acid content of naturally occurring and synthetic folates.

Significance to Bio-Medical Research and the Program of the Institute: The role of folic acid in metabolism has been well delineated. In contrast, little solid information is available about the function of polyglutamyl derivatives of folic acid despite the fact that the vitamin occurs in living organisms largely in these forms. Folic acid conjugase is an enzyme of nearly universal occurrence which seems to be responsible for the *in vivo* conversion of folic acid derivatives of high glutamic acid content (up to seven residues per molecule) to forms having fewer glutamyl residues. The study of this enzyme promises to be a fruitful means of elucidating both the mechanism and regulation of the production of the various polyglutamyl derivatives and thereby providing insight into their functions.

Honors and Awards: NONE

Publications: NONE

Serial No. NIAMD-LNE-15
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Structural analysis of the active and inactive portions of the eukaryotic genome.

Previous Serial Number: NONE

Principal Investigator: Dr. Gerald R. Reeck

Other Investigators: Dr. Robert T. Simpson and Dr. Herbert A. Sober

Cooperating Units: NONE

Man Years

Total:	1.0
Professional:	1.0
Other:	0

Project Description:

Objectives: To determine the physico-chemical basis for the functional differences between the transcribable portion and the remainder of the eukaryotic genome.

Methods Employed: Fractionation of chromatin is performed on a weak anion-exchange cellulose, ECTHAM-cellulose, which had been designed specifically for the chromatography of nucleic acids. Fractions are analyzed by quantitative protein determinations, gel electrophoresis, viscosity, circular dichroism spectroscopy, endogenous enzymatic activity, and melting profile.

Major Findings: Sonication at 20,000 cycles per second has been used to reduce the size of the chromatin molecules and thus increase the possibility of having homogeneous populations. By melting profile analysis, we have shown this somewhat drastic procedure produces, at most, only minimal effects on the chromatin beyond reduction in molecular weight. Simultaneously monitoring at 0.5° intervals, the absorbances at 260 nm of sonicated and unsonicated samples showed only very small differences.

Sonicated chromatin has been fractionated on ECTHAM cellulose into a series of fractions having a continuous spectrum of melting profiles, the weak binding material melting at a considerably higher temperature than unfractionated chromatin and the latest eluting material having much more low melting component than unfractionated chromatin. Intermediate fractions melt essentially like chromatin. Although there are not detectable quantitative differences in

total protein content in the various fractions, there are qualitative differences as revealed by gel electrophoresis. The tightly-bound material is strikingly enriched above unfractionated chromatin in a nonhistone of high molecular weight; in contrast, the weakly bound material lacks this protein. Roughly corresponding to the appearance of this protein is a decrease in the content of lysine-rich histone. No differences have been detected in the circular dichroic spectra of the fractions.

Attempts to determine which fractions contain the endogenous RNA polymerase of chromatin have not been entirely successful, but preliminary indications are that the tightly bound material may contain this activity and hence be the transcribable portion of the genome.

Significance to Bio-Medical Research and the Program of the Institute: Central to understanding genetic control in eukaryotic organisms is the acquisition of detailed knowledge of the structural basis for transcriptional capability of DNA in chromatin. Only with this information at hand will the fast accumulating body of facts from various control systems be fully interpretable. The studies are undertaken to provide this understanding of the structure of chromatin's transcribable component. Our results should furthermore allow us and other investigators to utilize the fractionation procedure in analysis of specific control systems in normal and disease states.

Awards and Honors: NONE

Publications: NONE

Serial No. NIAMD-LNE-16
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Purification of the Hurler corrective factor from normal human urine.

Previous Serial Number: NONE

Principal Investigator: Dr. Pamela Roddy

Other Investigator: Dr. Elizabeth Neufeld (A-LBM)

Man Years

Total:	1.0
Professional:	1.0
Other:	0

Project Description:

Objectives: To work out the conditions for the large-scale isolation and purification of the Hurler factor from normal human urine so that it can be used in replacement therapy of afflicted Hurler patients.

Methods Employed: Protein purification techniques including $AmSO_4$ precipitation, Sephadex G-200 column chromatography, CM-cellulose and hydroxy-apatite chromatography. The bioassay for the corrective ability of the protein factor is performed in cell cultures of Hurler fibroblasts obtained from biopsy of affected patients, and is measured by the cellular incorporation of radioactive SO_4.

Major Findings: Urine and plasma were investigated as potential sources of the protein corrective factor for the Hurler syndrome. It appears that urine is a richer source of this factor than is plasma in which it may exist in some cryptic form. Hurler factor has been purified from large pools of urine. Purification conditions are being worked out so that purification can eventually be handled by a contractor.

Significance to Bio-Medical Research and the Program of the Institute: Purified Hurler factor can be used to treat children with the Hurler syndrome, a genetic disease of mucopolysaccharide metabolism. At the present time, these children are being treated with some success by plasma therapy, but since urine is a much richer source of this protein factor, once purified, it will be more efficacious therapeutically.

Honors and Awards: None
Publications: None

ANNUAL REPORT SUMMARY

LABORATORY OF BIOCHEMISTRY AND METABOLISM

Carbohydrate Metabolism

A. Mucopolysaccharidoses. The abnormal mucopolysaccharide metabolism of fibroblasts from patients with genetic mucopolysaccharidoses is due to the absence of a specific protein or "factor". When this protein is supplied exogenously, the metabolism is restored to normal. As a result of recent studies, the following summary can be made. 1. The factor deficient in Sanfilippo fibroblasts of the A subtype has been shown to degrade the heparan sulfate that accumulates in those fibroblasts. The Sanfilippo A factor is most probably a heparan sulfate N-sulfatase. 2. The Hurler and Scheie syndromes had been previously shown to have a deficiency of, and to be correctible by the same protein - designated "Hurler factor". This factor has now been shown to be a α-L-iduronidase; consequently, the Hurler and Scheie syndromes are α-L-iduronidase deficiency diseases. 3. Normal fibroblasts in culture show an impairment in mucopolysaccharide degradation when the pH of medium is raised to 7.4 or higher. At pH 8, normal cells are not distinguishable from those with a mucopolysaccharidosis. They grow, however, remarkably well at the higher pH. [E. F. Neufeld]

B. Chitin and Glycogen Synthetase. In early studies a hypothesis was developed to explain the mechanism of initiation of the chitinous primary septum during cell division in yeast. Briefly, it was proposed that an activating factor would transform inactive chitin synthetase (zymogen) into active enzyme only at certain sites in the cell surface, thus explaining the localization of the chitin septum. A necessary assumption was the activating factor and the zymogen would reside in different organelles, but the evidence for this was only indirect. An effort was therefore made to isolate the organelles which contain the activating factor. In the supposition that these organelles would be similar or identical to the yeast cell vacuoles, the isolation of the latter was attempted. By a combination of "metabolic lysis" of spheroplasts and centrifugations in Ficoll discontinuous gradients it was possible to obtain in good yield a vacuole fraction of sufficient purity. This fraction was greatly enriched in chitin synthetase activating factor (about 20-fold on a protein basis) and was essentially free of zymogen. The latter was found in another, membrane--rich fraction, which was devoid of activating factor. It has thus been established that activating factor and zymogen are spatially separated in the cell. Studies on the further characterization of the isolated subcellular fractions are in progress.

The fact that trypsin can mimic the effect of the activating factor suggested that the latter might be a protease. This hypothesis has now been confirmed. This finding provided an assay for activating factor which is independent from the chitin synthetase system. The assay is now used in the further purification of the activating factor inhibitor. Recent studies

using acrylamide gel electrophoresis indicate that the inhibitor has a low molecular weight, about 15,000.

Studies on the purification of the different forms of glycogen synthetase have been pursued further. A major advance was the finding that the glucose-6-phosphate independent (I) and glucose-6-phosphate dependent (D) forms can be separated by DEAE chromatography. This is the first case in which I and D forms have been separated one from the other from any source. It has thus been possible to establish a correlation between glucose-6-phosphate dependence of the preparation and actual presence of each form. Another consequence has been the development of a purification method for the D form. [E. Gabib]

C. Particulate Enzymes of Carbohydrate Metabolism. The properties of glucose-6-phosphatase and related enzymes are different in rough and smooth endoplasmic reticular membranes. In membrane areas having attached ribosomes the enzyme is predominantly in an "active" configuration while that in the smooth membranes is in a less active or potential form.

A comparative kinetic study of the phosphorylation of the 5-carbon sugar alcohols by inorganic pyrophosphate and microsomal enzyme has been carried out. The four pentitols serve as acceptors for the phosphotransferase activity from PP_i at varying rates: D-arabitol > ribitol > L-arabitol > xylitol. In livers of fasted and diabetic rats the levels of enzyme activity are greatly elevated over those found in normal fed animals. The product of the enzymatic phosphorylation of ribitol has been isolated and characterized as D-ribitol-5-phosphate. [M. Stettin]

D. Inositol Biosynthesis. No isotope incorporation into inositol was observed when glucose-6-phosphate and NAD-4-^{3}H were incubated with purified testicular cyclase. As indicated above a negative result is inconclusive in defining the role of the coenzyme.

The butaneboronic acid derivatization reaction for the gas chromatography of carbohydrates has been extended to amino sugars and uronic acids. Thus neutral, basic, and acidic carbohydrates can be analyzed by this method, which is now being applied to the study of the hydrolysis of mucopolysaccharides by the Hunter and Hurler factors of Neufeld. The role of the Hurler factor as an iduronidase has been confirmed by this method.

The formation of neoinositol, an isomer of myoinositol, from mannose 6-phosphate by a soluble rat testis enzyme has confirmed the original observation of Sherman. The difficulty experienced by Sherman in separating neoinositol from mannose as the trimethylsilyl derivatives by gas chromatography has been overcome in this study by the use of the butaneboronates. [F. Eisenberg]

E. Glycoprotein Metabolism. A general method has been developed for the quantitative determination of the binding of glycoproteins to the plasma membranes of rat liver which is based upon the competitive inhibition of the binding phenomenon in an operationally irreversible system. Ulitizing this procedure, a quantitative estimate of the ability of the membranes to

bind α1 acid glycoprotein, fetuin, ceruloplasmin and transferrin, as well as their asialo- and agalacto-derivatives, has been made. The results obtained extend the previous qualitative observations in vivo and provide the basis for a systematic analysis of the structural determinants involved in the binding process.

Following the previously described identification of the hepatic plasma membranes as the major locus of binding for asialo-glycoproteins, subsequent investigation has revealed that certain other cellular organelles such as lysosomes, microsomes and golgi apparatus share this property. In each case, binding is eliminated by prior treatment with neuraminidase. Consequently, the role of membrane bound sialic acid in the binding process has been investigated. Preliminary findings support the hypothesis that membrane associated (or intrinsic) sialyl transferase constitutes an integral part of the binding mechanism.

In an attempt to extend the above observations to systems exhibiting macromolecular transport, as well as binding, normal liver cells have been grown in tissue culture. In contradistinction to freshly isolated, intact hepatocytes, the surface membranes of the cells grown in tissue culture no longer possess the ability to bind asialo-glycoproteins. Hepatoma cells, grown in tissue culture, are similarly incompetent. However, cells isolated directly from secondary hepatoma transplants do exhibit the binding phenomena although at a significantly reduced level. Current studies are directed at establishing a correlation between changes in the cell surface membranes and the loss of binding capacity. [G. Ashwell]

F. Immunoglobulin Biosynthesis. Incompletely synthesized heavy chains of immunoglobulin G were produced by rabbit lymph node cells incubated in the presence of 10^{-5} M puromycin. After cyanogen bromide treatment, such incomplete chains yielded peptides which overlapped with those derived from native heavy chains. Cells incubated with ^{3}H-puromycin produced radioactive materials which were precipitated with a serum specific for immunoglobulin G. Furthermore, reduced and alkylated ^{3}H-puromycin labeled immunoglobulins were smaller than heavy chains as judged by sodium dodecyl sulfate - polyacrylamide gel electrophoresis. Incomplete heavy chains were synthesized as well as secreted into the extracellular medium by lymph node cells incubated in the presence of puromycin.

Colostral immunoglobulin A was examined for possible subunits analogous to the immunoglobulin G-like subunit of immunoglobulin M. Reduction of such immunoglobulin A with dithiothreitol yielded three slower moving components on molecular sieve chromatography in the presence of physiological saline. One of the products was identified as light chain. Of the other two products, one appears to be composed of molecules having one each of heavy, light and secretory component chains. This structure best fits the data obtained from estimates of its molecular weight via column chromatography, from chain composition analysis and from the known molecular weight of its component polypeptide chains. Based on these same criteria, the remaining reduction product of IgA best fits a molecular structure composed of 3 heavy and 3 light chains. [M. Kern]

G. Hormone Dependent Differentiation of Mammary Gland. 1. Mammary epithelial cell differentiation, as effected by the interplay of insulin, corticosteroid(s), and prolactin, has been further studied in vitro. Emphasis has been placed on the synthesis of casein in relation to the fabrication and function of the rough endoplasmic reticulum (RER).

Minimal hormone requirements for the accumulation of the RER are insulin and corticosteroid. This is based on determinants of membrane--linked NADH-cytochrome C reductase and of RNA content of isolated RER. Accumulation of the enzyme is affected very little by insulin, modestly increased by hydrocortisone, and greatly enhanced by the combination of the two hormones. RNA content of RER is virtually unaltered by hydrocortisone, but is increased by insulin. This effect of insulin is, however, enhanced by the presence of hydrocortisone. Addition of hydrocortisone to an insulin-containing postmitotic system causes redistribution of free ribosomes to the membrane-bound variety without a net increase in ribosomes. In the absence of the steroid, insulin promotes development of an unstable RER which also is deficient in NADH-cytochrome C reductase.

Prolactin does not affect the membrane-linked enzyme. It does, however, stimulate the rate of RNA synthesis and increases the RNA content of the membrane system. In the absence of RER, prolactin also increases the rate of RNA synthesis, but this effect is not sustained.

Three lines of evidence strongly suggest that casein formation by these epithelial cells is dependent upon the presence of the RER that is induced by insulin and hydrocortisone. (a) There is close agreement between casein synthesis and RER formation as a function of hydrocortisone concentration. (b) RER formation precedes casein synthesis. (c) Casein formation requires sustained stimulation of RNA synthesis by prolactin, which, in turn, is dependent on the presence of RER.

2. Virgin mammary epithelial cells are dormant in terms of proliferation and development because they are insensitive to insulin and serum factors. Sensitivity to these agents is acquired by the second day of pregnancy in the mouse. Yet, the "insulin-apparatus" is already present in the virgin animal, since the mammary cells do respond to insulin--sepharose. The "apparatus", then, is masked in the nonpregnant animal. Microscopic examination of mixed suspensions of the cells and insulin--sepharose beads reveal that the two particulates do not form complexes with each other. It appears, therefore, that effective interaction between these target cells and insulin does not require tight binding, although collisions are necessary. [Y. Topper]

H. Enzymatic Utilization of Model Compounds. Continuing study of an aldehyde dehydrogenase from baker's yeast has led to information defining the effect of glycerol on enzymes. During the past decade this laboratory among others has become dependent on glycerol as a means of stabilizing enzymes which could not have been purified without this reagent. This stabilizing effect, more or less, duplicated by several polyhydric alcohols, is of such magnitude as to be able to name glycerol as

the "universal enzyme solvent". Allowing for hyperbole, this is an extraordinarily effective adjunct to enzyme purification when used at concentrations in the range of 20 to 50 per cent by volume. The effect of glycerol on aldehyde dehydrogenase is to change the conformation of the enzyme in a manner such that it binds both substrates, aldehyde and pyridine nucleotide, far better than when simply in aqueous solution. This conformational change is also measurable by the decreased reactivity of the enzyme with alkylating reagents and with trivalent arsenicals. There is now evidence that the phenomenon displayed by aldehyde dehydrogenase may be a general one resulting from the presence of glycerol.

An enzyme has been isolated in homogeneous form from rat liver which catalyzes the conjugation of glutathione with any of a variety of epoxides which bear the ethylene oxide group at the terminal portion of an aliphatic chain. The product is a thioether. Several such proteins are found in liver, all with similar if not identical specificity, differing only in their isoelectric point. Under appropriate circumstances, each of at least 3 such proteins may be converted to a single protein which has the same specificity but a greater turnover number. [W.B. Jakoby]

I. Thermodynamic and Kinetic Studies of Enzymes. As predicted theoretically, fast changes in absorbance have been detected in kinetic studies of the unfolding of ribonuclease A and horse heart metmyoglobin. It has originally been postulated that such changes would be detected when the protein was mainly in its folded form. Experimentally the opposite behavior is found. The fast change becoming more pronounced as the protein becomes less stable, this is compatible with theory, if the observed absorbance change occurs at some fast change in the unfolding reaction, before the slow nucleation step has been reversed. The variation of the fast change with pH and temperature contains information on the number of such fast steps in the reaction.

Characteristic absorbance changes accompanying the conversion of pepsinogen into pepsin have been observed. Where comparable, these changes parallel the appearance of pepsin and show similar dependence on ionic strength, pH and protein concentration. A fast conformational change has been observed in pepsinogen, at low pH, which may be interpreted as bringing the molecule into an intermediate state where self-proteolysis may occur.

Kinetic and equilibrium studies on protein unfolding are being continued, to determine the nature of the intermediate states seen in the unfolding reaction and to determine the relationship of the thermally unfolded form of the molecule to the randomly coiled form. This latter form is probably a better approximation to the newly synthesized form of the protein, in the cell. [P. McPhie]

J. Enzyme Induction. 1. The susceptibility of actively accumulated substrate pools to cold shock loss has been shown to be independent of the age of the culture and of the absolute osmolarity or buffer composition of the incubation or growth medium. A report concluding, in contrast to these studies, that cold shock causes

negligible loss of accumulated thiomethyl galactoside, has been shown to have been based on studies with a strain of E. coli which is particularly resistant to cold shock. Efforts to adduce evidence that cold shock leads to the formation of transient hydrophyllic channels by demonstrating the non-catalytic entry of labeled substrates have been unrewarding.

2. The crystalline compound derived from heptaacetylmethylgalacturonate-thio-galactoside has been shown to specifically interfere with lac permease having no effect on amino acid accumulation. [I. Leder]

K. Enzymatic Reduction of Disulfide Linkages in Mammalian Cells. The current study of the subcellular distribution of enzymes catalyzing thiol-disulfide exchange reactions (thiol-disulfide transhydrogenases) in rat liver has yielded the following information: 1) The post-microsomal (soluble) fraction contains a single activity associated with the catalysis of interchange reactions between glutathione (GSH) and substrates. 2) The microsomal fraction possesses two additional transhydrogenase activities which are distinct from the supernatant enzyme. While both of these microsomal enzymes possess GSH-insulin transhydrogenase activity, only one is capable of catalyzing interchange reactions between GSH and small disulfides. 3) The mitochondrial and lysosomal fractions appear to be inactive with respect to the above enzyme activities. [F. Tietze]

L. Biosynthesis of Sulfur Containing Nucleotides. Natural 4-thiouridine has invariably been found as the 8th nucleotide from the 5' end of the tRNA molecule. Thiolation was carried out on pure species of phenylalanine tRNA from E. coli (using a preparation which was already partially thiolated in the 8th position) and from baker's yeast (which has uridine in the 8th position). The enzymatically thiolated materials were treated with ribonuclease and the digests separated on benzoylated DEAE-cellulose columns. In both cases, although approximately half of the sulfur had been introduced into the 8th position, other sites on the minor loop or the T4C loop were also labeled.

The first enzyme in the thiolation system, named Factor A, requires tRNA, ATP and Mg^{++}. Studies are under way to characterize the intermediate arising from this interaction. Preliminary results indicate that there is no phosphate or pyrophosphate exchange associated with the reaction, and binding studies thus far demonstrate no adenylate uptake by tRNA or protein.

The second enzyme in the system, a pyridoxal-phosphate (PLP) enzyme, has been further purified, and the apoenzyme prepared by hydroxylapatite chromatography. The enzyme is inactivated by incubation with amino acids, except when α-keto acids are simultaneously present, and may be reactivated by incubation with PLP. [M. N. Lipsett]

Serial No. NIAMD-LBM-1
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Role of Hepatic Plasma Membranes in the Turnover of Circulating Glycoproteins.

Previous Serial Number: NIAMD-LBM-1

Principal Investigator: Dr. Gilbert Ashwell

Other Investigators: Dr. Jean Hickman, Research Chemist
Dr. Lee Van Lenten, Research Chemist
Mr. William Pricer, Research Chemist

Cooperating Units: None

Man Years

Total:	4-1/2
Professional:	4
Other:	1/2

Project Description:

Objectives:

The major objective of this project has been to ascertain the structural determinants of binding of the circulating glycoproteins by the plasma membranes of the liver.

Methods Employed:

Hepatic plasma membranes are isolated by conventional methods of cell fractionation employing sucrose density gradient centrifugation. Utilizing this tissue, an inhibition assay has been developed to provide a quantitative estimate of the binding efficiency of various glycoproteins and their asialo-derivatives.

Major Findings:

A general method has been developed for the quantitative determination of the binding of glycoproteins to the plasma membranes of rat liver which is based upon the competitive inhibition of the binding phenomenon in an operationally irreversible system. Utilizing this procedure, a quantitative estimate of the ability of the membranes to bind α_1 acid

Major Findings: (continued)

glycoprotein, fetuin, ceruloplasmin and transferrin, as well as their asialo- and agalacto-derivatives, has been made. The results obtained extend the previous qualitative observations in vivo and provide the basis for a systematic analysis of the structural determinants involved in the binding process. [Dr. L. Van Lenten]

Following the previously described identification of the hepatic plasma membranes as the major locus of binding for asialo-glycoproteins, subsequent investigation has revealed that certain other cellular organelles such as lysosomes, microsomes and golgi apparatus share this property. In each case, binding is eliminated by prior treatment with neuraminidase. Consequently, the role of membrane bound sialic acid in the binding process has been investigated. Preliminary findings support the hypothesis that membrane associated (or intrinsic) sialyl transferase constitutes an integral part of the binding mechanism. [Mr. Wm. Pricer]

In an attempt to extend the above observations to systems exhibiting macromolecular transport, as well as binding, normal liver cells have been grown in tissue culture. In contradistinction to freshly isolated, intact hepatocytes, the surface membranes of the cells grown in tissue culture no longer possess the ability to bind asialo-glycoproteins. Hepatoma cells, grown in tissue culture, are similarly incompetent. However, cells isolated directly from secondary hepatoma transplants do exhibit the binding phenomena although at a significantly reduced level. Current studies are directed at establishing a correlation between changes in the cell surface membranes and the loss of binding capacity. [Dr. J. Hickman]

Proposed Course of Project:

1. It is clear that, although the presence of a terminal galactose residue of the carbohydrate chain of the glycoproteins is a major determinant of binding, this parameter is neither unique nor sufficient to explain the broad spectrum of binding behavior. Consequently, application of the in vitro binding assay will be used to provide further information on the additional structural determinants of binding i.e. the role of the protein backbone, the participation of critical amino acids in isolated glycopeptides and the structural specificity of the individual carbohydrate chains.

2. Attempts to isolate and characterize the receptor sites on the plasma membranes are currently in progress.

3. Extension of the studies on tissue culture and hepatoma cells will be carried out in an attempt to correlate cell surface changes with altered binding capacity.

Publications:

Pricer, W. E. and Ashwell, G.: The binding of desialylated glycoproteins by plasma membranes of rat liver. J. Biol. Chem. 246: 4825-4833, 1971.

Snyder, S. and Ashwell, G.: Quantitation of specific serum glycoproteins in malignancy. Clin. Chim. Acta 34: 449-455, 1971.

Ashwell, G. and Morell, A. G.: Galactose: A cryptic determinant of glycoprotein catabolism. In Jamieson, G. A. and Greenwalt, T. J. (Eds.): Glycoproteins of blood cells and plasma. Philadelphia, J. B. Lippincott Co., 1971, pp. 173-189.

Vaitukaitis, J. L., Hammond, J., Ross, G. T., Hickman, J., and Ashwell, G.: A new method of labeling human chorionic gonadotropin for physiological studies. J. Clin. Endocr. 32: 290-293, 1971.

Ashwell, G. and Hickman, J.: The chemistry of the unique carbohydrates of bacterial lipopolysaccharides. In Weinbaum, G., Kadis, S., and Ajl, S. J. (Eds.): Microbiol Toxins. New York, Academic Press, 1971, pp. 235-266.

Vaitukaitis, J. L., Sherins, R., Ross, G. T., Hickman, J., and Ashwell, G.: A method for the preparation of radioactive FSH with presevation of biologic activity. Endocrinology 89: 1356-1360, 1971.

Van Hall, E. V., Vaitukaitis, J. L., Ross, G. T., Hickman, J., and Ashwell, G.: Effects of progressive desialylation on the rate of disappearance of immunoreactive HCG from plasma in rats. Endocrinology 89: 11-15, 1971.

Van Lenten, L., and Ashwell, G.: The binding of desialylated glycoproteins by plasma membranes of rat liver: Development of a quantitative inhibition assay. J. Biol. Chem., in press.

Serial No. NIAMD-LBM-2
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Enzymatic Utilization of Model Compounds

Previous Serial Number: NIAMD-LBM-2

Principal Investigator: Dr. William B. Jakoby

Other Investigators: Dr. Shelby L. Bradbury, Staff Fellow
Dr. Thorsten Fjellstedt, Staff Fellow

Cooperating Units: None

Man Years

Total:	4
Professional:	3
Other:	1

Project Description:

Objectives:

These investigations are concerned with the reactivity of a variety of chemical groupings in enzyme-catalyzed reactions. Choices are such as to lead to investigations of the general phenomena of enzyme mechanisms. Particular attention is presently directed to a versatile detoxification mechanism by which addition compounds of glutathione are metabolized in preparation for excretion by the kidney.

Methods Employed:

The approach is that of purification of the chosen enzyme and its study by chemical and physical methods.

Major Findings:

These studies have continued a two-fold emphasis on the metabolic utilization of a variety of model compounds bearing specific functional groups and, as well, on those features of proteins responsible for specific catalysis.

Major Findings (continued):

Continuing study of an aldehyde dehydrogenase from baker's yeast has led to information defining the effect of glycerol on enzymes. [S. Bradbury] During the past decade this laboratory among others has become dependent on glycerol as a means of stabilizing enzymes which could not have been purified without this reagent. The stabilizing effect, more or less, duplicated by several polyhydric alcohols, is of such magnitude as to be able to name glycerol as the "universal enzyme solvent." Allowing for hyperbole, this is an extraordinarily effective adjunct to enzyme purification when used at concentrations in the range of 20 to 50 per cent by volume. The effect of glycerol on aldehyde dehydrogenase is to change the conformation of the enzyme in a manner such that it binds both substrates, aldehyde and pyridine nucleotide, far better than when simply in aqueous solution. This conformational change is also measurable by the decreased reactivity of the enzyme with alkylating reagents and with trivalent arsenicals. There is now evidence that the phenomenon displayed by aldehyde dehydrogenase may be a general one resulting from the presence of glycerol.

An enzyme has been isolated in homogeneous form from rat liver which catalyzes the conjugation of glutathione with any of a variety of epoxides which bear the ethylene oxide group at the terminal portion of an aliphatic chain. [T. Fjellstedt] The product is a thioether. Several such proteins are found in liver, all with similar if not identical specificity, differing only in their isoelectric point. Under appropriate circumstances, each of at least 3 such proteins may be converted to a single protein which has the same specificity but a greater turnover number. [T. Fjellstedt]

Significance to NIAMD Research:

Studies of enzyme mechanism and of patterns of metabolism relate directly to the fundamentals of all biological activity. Work on epoxides is intimately related to a general mechanism of aromatic oxidation as well as to the biological problem of detoxification of noxious compounds.

Proposed Course of Project:

It is expected that investigations dealing with the above-noted projects will be continued and a study of other modal reactions will be initiated.

Publications:

Bradbury, S. L. and Jakoby, W. B.: Ligand interactions with yeast aldehyde dehydrogenase. J. Biol. Chem. 246: 6929-6932, 1971.

Bradbury, S. L. and Jakoby, W. B.: Glycerol induced conformational change: Aldehyde dehydrogenase as a model. Fed. Proc. 31: 842-843, 1972.

Jakoby, W. B. and Fjellstedt, T. A.: Epoxidases. In Boyer, P. D. (Ed.): The Enzymes. New York, Academic Press, Vol. 7, 3ed., in press.

Bradbury, S. L. and Jakoby, W. B.: Glycerol as an enzyme stabilizing agent; Effect on sulfhydryl groups of aldehyde dehydrogenase. J. Biol. Chem. 247: in press.

Serial No. NIAMD-LBM-3
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on the Synthesis and Degradation of Nucleic Acids

Previous Serial Number: NIAMD-LBM-3

Principal Investigator: Dr. Maxine F. Singer

Other Investigators: Dr. Carol Letendre, Staff Fellow
Dr. Randall K. Holmes, Surgeon

Cooperating Units: Professor Ernest Winocour
Department of Genetics
Weizmann Institute of Science
Rehovot, Israel

Man Years

Total:	3
Professional:	3
Other:	

Project Description:

Objectives:

To continue studies on the structure of the enzyme polynucleotide phosphorylase, particularly as relates to catalysis of chain initiation, and the role of sulfhydryl groups in functional properties of the enzyme.

To study the apparent inhibition of RNase II of Escherichia coli by ATP.

To initiate studies on nucleic acid metabolism of animal viruses.

Methods Employed:

To a large degree both our preparative and analytical work depends on the use of enzymes as specific reagents. The purely chemical techniques are of limited value with nucleic acids. Instead, a large inventory of highly specific enzymes is maintained. The enzymes are either prepared in the laboratory or in some cases, obtained commercially.

Methods Employed: (continued)

Disk electrophoresis on polyacrylamide gels is used extensively both in mechanism studies and protein structure studies. Electrophoresis on acrylamide gels in the presence of sodium dodecylsulphate is also used.

Protein structure studies utilize the analytical ultracentrifuge and amino acid analyzer, as well as specific chemical and enzymic modification of the protein.

Studies with the animal virus involve standard tissue culture techniques for mammalian cells. Investigation of DNA metabolism utilizes ultracentrifugation both in gradients and by equilibrium techniques, as well as nucleic acid hydridization.

In addition, standard biochemical, radiochemical, and physical methods were used.

Major Findings:

1. Polynucleotide phosphorylase: Extensive studies of alkylation of enzyme sulfhydryls by N-ethylmaleimide under a variety of conditions have failed to give a consistent pattern of results. These inconsistencies have led us to conclude that the primer-independent enzyme must be a flexible molecule whose polymer initiating activity is a function of the state of oxidation of crucial sulfhydryl groups. Isolation and storage methods available at present do not permit maintenance of a single structural form. Such difficulties also account for variation in the actual number of sulfhydryl groups alkylated under different conditions. (It is clear, as we observed earlier, however, that alkylation of less than half of the 10 cysteine residues in primer-independent enzyme, leads to formation of an enzyme form that is highly stimulated by oligonucleotide primer.) Substrates such as poly adenylic acid, added or polymerized *in situ*, partially protect against loss of activity following reduction and alkyation even though the number of sulfhydryls actually alkylated remains unchanged. Recent experiments in progress include the isolation of the C^{14}-carboxymethyl cysteine-containing peptides from tryptic digests of the primer-dependent form of the enzyme. In a small-scale, preliminary experiment, one such peptide was isolated confirming ultracentrifuge data which suggested four identical subunits.

2. Inhibition of *Escherichia coli* RNase II: In the course of earlier experiments on the putative RNase V of *E. coli* we noted that in crude extracts, RNase II activity was inhibited by ATP. Experiments with highly purified RNase II indicated that inhibition required ATP as well as a protein factor present in the crude extracts. Purification of this protein factor, and study of the products of the inhibited reaction showed a trivial explanation for the inhibitor. The purified "inhibitor" proved

Major Findings: (continued)

to be nucleoside monophosphate kinase, which, in the presence of ATP, converted the RNase II product, 5'-AMP, to ADP. Under the standard procedure for determining production of alcohol-soluble 5'-AMP from poly A, the ADP is precipitated to a significant extent. The specificity of the highly purified kinase indicates that it is specific for adenine nucleotides, either ribose or deoxyribose.

3. Animal viruses: This work has been carried out in the laboratory of Professor Ernest Winocour, Department of Genetics, Weizmann Institute of Science.

Conditions leading to incorporation of host genome sequences into duplex circular DNA of SV40 progeny upon productive infection of BSC-1 cells with SV40 virus were determined previously in this laboratory. One such condition, multiple passage of plaque purified virus at high multiplicity of infection, has now been investigated more thoroughly. Acquisition of host sequences occurs with two different lines of SV40. Within one line, different clones give rise, after multiple passage, to defective virus containing different amounts of host sequence. One such population of defective virus appears to contain host sequences in at least 90 percent of the duplex circular DNA. Fragmentation of the DNA and hybridization suggests that host sequences may account for about 10 percent of the DNA. Specific degradation of the duplex circular DNA by a bacterial restriction enzyme (compared to degradation of DNA from plaque purified virus) indicates major modifications in the genome.

Significance to NIAMD Research:

The importance of understanding the mechanism of biological polymerization reactions, in this instance polymerization of nucleotides, is clear. Recent experiments in virology have emphasized this point. The work on the detailed mechanism of polynucleotide phosphorylase catalyzed reactions is therefore of importance, the more so because of the complexity of the enzyme protein itself. Furthermore, recent publications indicate the presence of this enzyme in animal cells, thus widening interest.

The proposed studies in animal virology are consistent with wide-spread feeling that complex animal systems are now amenable to detailed biochemical investigation. It is hoped that such studies will lead to increased understanding not only of the mechanism of viral action, but also of the properties of the cells they infect.

Proposed Course of Project:

Investigation of the structure of polynucleotide phosphorylase will continue. In addition, studies on the mechanism of initiation of polymerization will be started.

Work on DNA replication of Simian Virus 40 will start in Bethesda. This will necessitate setting up tissue culture lines and applying techniques learned at the Weizmann Institute. As a corollary to this work a variety of DNases, specific for single strands, will be investigated as tools to improve currently available DNA-DNA hybridization techniques.

Publications:

Holmes, R. K. and Singer, M. F.: Inability to detect RNase V in Escherichia coli and comparison of other ribonucleases before and after infection with coliphage T7. Biochem. Biophys. Res. Commun. 44: 837-843, 1971.

Chou, J. Y. and Singer, M. F.: Deoxyadenosine diphosphate as a substrate and inhibitor of polynucleotide phosphorylase of Micrococcus luteus. I. Deoxyadenosine diphosphate as a substrate for polymerization and the exchange reaction with inorganic ^{32}P. J. Biol. Chem. 246: 7486-7496, 1971.

Chou, J. Y. and Singer, M. F.: Deoxyadenosine diphosphate as a substrate and inhibitor of polynucleotide phosphorylase of Micrococcus luteus. II. Inhibition of the initiation of adenosine diphosphate polymerization by deoxyadenosine diphosphate. J. Biol. Chem. 246: 7497-7504, 1971.

Chou, J. Y. and Singer, M. F.: Deoxyadenosine diphosphate as a substrate and inhibitor of polynucleotide phosphorylase of Micrococcus luteus. III. Copolymerization of adenosine diphosphate and deoxyadenosine diphosphate. J. Biol. Chem. 246: 7505-7513, 1971.

Holmes, R. K. and Singer, M. F.: An ATP-dependent inhibitor of E. coli RNase II. Fed. Proc. 31: 471, 1972.

Serial No. NIAMD-LBM-4
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on Naturally Occurring Sulfur Nucleotides: Identification, Chemistry, Biosynthesis and Biological Significance

Previous Serial Number: NIAMD-LBM-4

Other Investigators: Dr. Beatrice Chen, Visiting Associate

Cooperating Units: None

Man Years

Total:	2.4
Professional:	1.8
Other:	0.6

Project Description:

Objectives:

The cysteine-4-thiouridine sulfur transferase system is unique in several respects. First, it is a two-enzyme system leading to a single base modification in tRNA. Secondly, its specificity apparently resides in some as yet undiscovered aspect of tRNA tertiary structure. The purpose of this investigation is to gain information about that tertiary structure, and incidentally about the role of 4-thiouridine in the tRNA molecule. Observations on the pyridoxal-phosphate-requiring enzyme system itself are also of interest.

Methods Employed:

Enzyme purification has been carried out by standard techniques of salt fractionation, phase separation and column chromatography. Separation of oligonucleotide digests of tRNA were carried out on benzoylated DEAE columns. Electrophoresis and DEAE-cellulose columns resolved nucleotide mixtures. The cell-free *in vitro* systems of Zubay and of Pastan were used to evolve the tRNA-requiring protein synthesis system.

Major Findings:

1. Natural 4-thiouridine has invariably been found as the 8th nucleotide from the 5' end of the tRNA molecule. Thiolation was carried out on pure species of phenylalanine tRNA from E. coli (using a preparation which was already partially thiolated in the 8th position) and from baker's yeast (which has uridine in the 8th position). The enzymatically thiolated materials were treated with ribonuclease and the digests separated on benzoylated DEAE-cellulose columns. In both cases, although approximately half of the sulfur had been introduced into the 8th position, other sites on the minor loop or the T4C loop were also labeled.

2. Studies on the function of 4-thiouridine in tRNA require not only a tRNA species thiolated in a specific and known position, but also a tRNA-dependent test system. Dr. Chen has been working on a modification of the DNA-dependent β-galactosidase-forming system to introduce an absolute tRNA requirement. This system will serve as a test system for the function of 4-thiouridine in ribosomal binding and protein formation.

3. The first enzyme in the thiolation system, named Factor A, requires tRNA, ATP and Mg^{++}. Studies are under way to characterize the intermediate arising from this interaction. Preliminary results indicate that there is no phosphate or pyrophosphate exchange associated with the reaction, and binding studies thus far demonstrate no adenylate uptake by tRNA or protein. These results must be checked by alternative methods.

4. The second enzyme in the system, a pyridoxal-phosphate (PLP) enzyme, has been further purified, and the apoenzyme prepared by hydroxylapatite chromatography. The enzyme is inactivated by incubation with amino acids, except when α-keto acids are simultaneously present, and may be reactivated by incubation with PLP. This system is apparently analogous to the aspartate β-decarboxylase studied by E. Miles. Borohydride also destroys activity, presumably by reducing the PLP, but does not form a covalent bond between protein and PLP. This may indicate that the sulfurtransferase is not linked to PLP through a Schiff base type of bonding, which would be quite unusual.

Significance to NIAMD Research:

A specific conformation of tRNA is apparently crucial if the molecule is to be thiolated only in the natural 8th position from the 5' end. Thus this sulfurtransferase system offers a new probe for tertiary structure in tRNA.

Proposed Course of Research:

Further work on the correlation between tertiary tRNA structure and availability of uridine residues for thiolation will be carried out. If conditions are found which will allow only a single substitution in the 8th position, detailed work on native and thiolated yeast phenylalanine tRNA will be carried out using several test systems, including the one developed by Dr. Chen.

Factor A is close to homogeneity, and efforts will continue to obtain both enzymes in pure form. Physical studies of enzyme-enzyme or enzyme-tRNA complexes are planned.

A preliminary survey is contemplated to see whether the synthesis of other known thionucleotides proceeds by way of specific activating enzymes, similar to Factor A, followed by an actual thiolation step mediated by a common enzyme, the PLP-containing sulfurtransferase which participates in 4-thiouridine formation.

Publications:

Lipsett, M. N.: Biosynthesis of 4-Thiouridylate: Participation of a sulfurtransferase containing pyridoxal-5'-phosphate. J. Biol. Chem. 247: 1458-1461, 1972.

Serial No. NIAMD-LBM-5
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Biosynthesis of Storage and Structural Polysaccharides and Its Regulation

Previous Serial Number: NIAMD-LBM-5

Principal Investigator: Dr. Enrico Cabib

Other Investigators: Dr. Kuo-Ping Huang, Visiting Fellow
Dr. Rodney Ulane, Staff Fellow

Cooperating Units: None

Man Years

Total: 4
Professional: 3
Other:

Project Description:

Objectives:

This project attempts to make use of polysaccharide biosynthesis, as a tool in the study of certain fundamental cellular processes, i.e., 1) the mechanisms by which morphological changes are initiated in the course of cell development, as exemplified by the formation of a septum in the yeast cell; 2) the regulatory systems for the biosynthesis of storage material, using glycogen in yeast as a model.

Methods Employed:

Several strains of yeast and their mutants are used as experimental organisms.

Each problem is investigated at the molecular, subcellular and cellular levels. Thus, in addition to the conventional methods of enzymology, gradient centrifugation is used to isolate cell organelles and to determine the intracellular distribution of the different component of the system under study. Furthermore, these observations are correlated with others made at the cellular level, using both biochemical methods (variations in enzymes and metabolites) and light or electron microscopy (direct observation or autoradiography).

Major Findings:

1. In early studies a hypotesis was developed to explain the mechanism of initiation of the chitinous primary septum during cell division in yeast. Briefly, it was proposed that an activating factor would transform inactive chitin synthetase (zymogen) into active enzyme only at certain sites in the cell surface, thus explaining the localization of the chitin septum. A necessary assumption was the activating factor and the zymogen would reside in different organelles, but the evidence for this was only indirect. An effort was therefore made to isolate the organelles which contain the activating factor. In the supposition that these organelles would be similar or identical to the yeast cell vacuoles, the isolation of the latter was attempted. By a combination of "metabolic lysis" of spheroplasts and centrifugations in Ficoll discontinuous gradients it was possible to obtain in good yield a vacuole fraction of sufficient purity. This fraction was greatly enriched in chitin synthetase activating factor (about 20-fold on a protein basis) and was essentially free of zymogen. The latter was found in another, membrane-rich fraction, which was devoid of activating factor.

It has thus been established that activating factor and zymogen are spatially separated in the cell. Studies on the further characterization of the isolated subcellular fractions are in progress.

The fact that trypsin can mimic the effect of the activating factor suggested that the latter might be a protease. This hypothesis was confirmed, when it was found that preparations of the activating factor attack the general protease substrate "Azocoll" (a commercial preparation from cowhide), and that this activity is completely inhibited by the heat-stable, proteinaceous inhibitor of the activating factor, which was previously purified from yeast cells. This finding provided us with an assay for activating factor, which is independent from the chitin synthetase system. The assay is now used in the further purification of the activating factor inhibitor. Recent studies using acrylamide gel electrophoresis indicate that the inhibitor has a low molecular weight, about 15,000. The same technique is being used in the preparative scale in order to further purify the inhibitor and to obtain information about its structure and properties.

2. Studies on the purification of the different forms of glycogen synthetase have been pursued further. A major advance was the finding that the glucose-6-phosphate independent (I) and glucose-6-phosphate dependent (D) forms can be separated by DEAE chromatography. This is the first case in which I and D forms have been separated one from the other from any source. It has thus been possible to establish a correlation between glucose-6-phosphate dependence of the preparation and actual presence of each form.

Major Findings: (Continued)

Another consequence has been the development of a purification method for the D form. The procedure consists in passing a crude preparation of the I form of the enzyme through a DEAE column. The glucogen synthetase (I) peak also contains glycogen synthetase kinase, the enzyme which transforms I into D form. By incubating the peak fractions with ATP and Mg^{++} the transformation is effected and the enzyme, now mainly in the D form, is submitted again to DEAE chromatography. Most of the protein emerges in the same position as in the first column, but the D enzyme has moved to a new position and is thus greatly purified. This method is now combined with those previously developed, in order to obtain pure preparations of both forms.

Significance to NIAMD Research:

The findings related to septum formation in yeast promise to provide for the first time the detailed molecular mechanism for the triggering of a morphological change in the cell. It is hoped that this case will provide a useful model for the general processes of differentiation and cell development.

The regulation of glycogen synthesis, on the other hand, is a model for the problem of energy distribution and storage in the cell. As was remarked in other occasions, there is a striking parallelism between these regulatory mechanisms, as observed in yeast and in mammalian cells. Furthermore, the possibilities of biochemical and genetic experimentation provided by a unicellular organism make of yeast a unique material for these studies.

Proposed Course of Project:

The recent discoveries made on the localization of the chitin synthetase activating factor and on the nature of its enzymatic activity pave the way for additional studies of its function in the cell and of its purification and detailed characterization.

Efforts will be made to improve simultaneously the purification of zymogen and activating factor inhibitor, in order to increase our knowledge of the complete system.

Using the newly found tools for the purification of glycogen synthetase, the isolation of the two forms of the enzyme will be pursued further.

Publications:

Cabib, E. and Farkas, V.: The Control of Morphogenesis: An enzymatic mechanism for the initiation of septum formation in yeast. Proc. Nat. Acad. Sci. U.S. 68: 2052-2056, 1971.

Serial No. NIAMD-LBM-6
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Thermodynamic and Kinetic Studies of Protein Structure and Enzymic Mechanisms

Previous Serial Number: NIAMD-LBM-6

Principal Investigator: Dr. Peter McPhie

Other Investigators: Dr. Murray Summers, Staff Fellow

Cooperating Units: None

Man Years

Total:	1.75
Professional:	1.75
Other:	

Project Description:

Objectives:

A simple sequential model of protein folding has recently been proposed. It is hoped to extend recent observations which are compatible with this theory, both to place it on a firmer basis and to obtain more definite information on the actual mechanism of unfolding and refolding.

A convenient spectrophotometric assay of the pepsinogen-pepsin activation has recently been described. It is hoped to use this assay to obtain information on the actual events accompanying the conversion of the inactive zymogen into the proteolytic enzyme. Information on the mechanism of the enzyme itself may also be revealed in these studies.

Methods Employed:

Visible and ultraviolet spectroscopy in both manual and recording instruments. Fast kinetic experiments are performed in a combined T-jump-stop flow apparatus obtained from the American Instrument Company. Slower reactions are studied in the recording spectrophotometer in the "slow-T-jump" device, described previously.

Major Findings:

As predicted theoretically, fast changes in absorbance have been detected in kinetic studies of the unfolding of ribonuclease A and horse heart metmyoglobin. It had originally been postulated that such changes would be detected when the protein was mainly in its folded form. Experimentally the opposite behavior is found. The fast change becoming more pronounced as the protein becomes less stable. This is compatible with theory, if the observed absorbance change occurs at some fast change in the unfolding reaction, before the slow nucleation step has been reversed. The variation of the fast change with pH and temperature contains information on the number of such fast steps in the reaction.

Characteristic absorbance changes accompanying the conversion of pepsinogen into pepsin have been observed. Where comparable, these changes parallel the appearance of pepsin and show similar dependence on ionic strength, pH and protein concentration. A fast conformational change has been observed in pepsinogen, at low pH, which may be interpreted as bringing the molecule into an intermediate state where self-proteolysis may occur.

Proposed Course of Study:

Kinetic and equilibrium studies on protein unfolding are being continued, to determine the nature of the intermediate states seen in the unfolding reaction and to determine the relationship of the thermally unfolded form of the molecule to the randomly coiled form. This latter form is probably a better approximation to the newly synthesized form of the protein, in the cell.

Further studies on pepsinogen and pepsin, on the activation reaction and their interactions with substrates, inhibitors and other ligands should give more information on the molecular basis of the activation process and the active site of the enzyme.

Publications:

McPhie, P.: The nature of the acid transition of ribonuclease A. J. Biol. Chem. 246: 5537-5538, 1971.

Tsong, T. Y., Baldwin, R. L., and McPhie, P.: A sequential model of nucleation-dependent protein folding: Kinetic studies of ribonuclease A. J. Mol. Biol. 63: 453-475, 1972.

McPhie, P.: pH Dependence of the thermal unfolding of ribonuclease A. Biochemistry 11: 879-883, 1972.

McPhie, P.: A spectrophotometric investigation of pepsinogen-pepsin conversion. in press

Serial No. NIAMD-LBM-7
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Hormone-Dependent Differentiation of Mammary Gland In Vitro.

Previous Serial Number: NIAMD-LBM-7

Principal Investigator: Dr. Yale J. Topper

Other Investigators: Dr. Ida S. Owens, Dr. Takami Oka and Dr. Barbara K. Vonderhaar

Cooperating Units: None

Man Years:

Total: 4
Professional: 3
Other: 1

Project Description:

Objectives - To study the molecular and cytological phenomena involved in development of mammary gland.

Methods Employed - Mammary gland explants or mammary epithelial cells are cultured in synthetic media. Synthesis of casein is followed by isolation after the tissue has been pulsed with radioactive precursors. Synthesis of α-lactalbumin is followed by measuring the formation of lactose in tissue extracts. DNA and RNA synthesis are studied using conventional methods. Cell proliferation is determined by cell counts. The accumulation of α-aminoisobutyric acid (AIB) is followed with the use of ^{14}C-AIB or ^{3}H-AIB.

Major Findings - A. Mammary epithelial cell differentiation, as effected by the interplay of insulin, corticosteroid(s), and prolactin, has been further studied in vitro. Emphasis has been placed on the synthesis of casein in relation to the fabrication and function of the rough endoplasmic reticulum (RER).

Minimal hormone requirements for the accumulation of the RER are insulin and corticosteroid. This is based on determinations of membrane-linked NADH-cytochrome C reductase and of RNA content of isolated RER. Accumulation of the enzyme is affected very little by insulin, modestly increased by hydrocortisone, and greatly enhanced by the combination of the two hormones. RNA content of RER is virtually unaltered by hydrocorti-

sone, but is increased by insulin. This effect of insulin is, however, enhanced by the presence of hydrocortisone. Addition of hydrocortisone to an insulin-containing postmitotic system causes redistribution of free ribosomes to the membrane-bound variety without a net increase in ribosomes. In the absence of the steroid, insulin promotes development of an unstable RER which also is deficient in NADH-cytochrome C reductase.

Prolactin does not affect the membrane-linked enzyme. It does, however, stimulate the rate of RNA synthesis and increases the RNA content of the membrane system. In the absence of RER, prolactin also increases the rate of RNA synthesis, but this effect is not sustained.

Three lines of evidence strongly suggest that casein formation by these epithelial cells is dependent upon the presence of the RER that is induced by insulin and hydrocortisone. (a) There is close agreement between casein synthesis and RER formation as a function of hydrocortisone concentration. (b) RER formation precedes casein synthesis. (c) Casein formation requires sustained stimulation of RNA synthesis by prolactin, which, in turn, is dependent on the presence of RER.

B. Virgin mammary epithelial cells are dormant in terms of proliferation and development because they are insensitive to insulin and serum factors. Sensitivity to these agents is acquired by the second day of pregnancy in the mouse. Yet, the "insulin-apparatus" is already present in the virgin animal, since the mammary cells do respond to insulin-sepharose. The "apparatus", then, is masked in the non-pregnant animal. Microscopic examination of mixed suspensions of the cells and insulin-sepharose beads reveal that the two particulates do not form complexes with each other. It appears, therefore, that effective interaction between these target cells and insulin does not require tight binding, although collisions are necessary.

Significance to Bio-medical Research and the Program of the Institute - During development of multicellular organisms the progenitors of some types of target cells which respond to different hormones are themselves not sensitive to the hormones. The system described above may be a useful model for studying developmental events which result in the conversion of hormone-insensitive into hormone-sensitive cells. Extension of our studies with insulin-sepharose may further clarify the dynamics of insulin action and those of other polypeptide hormones.

Proposed Course of Project - The molecular mechanism involved in the acquisition of insulin-sensitivity will be studied. Also, the reason why DNA synthesis is required for differentiation of virgin mammary epithelial cells will be investigated.

Honors and Awards: None

Publications:

Topper, Y. J., Friedberg, S. H. and Oka, T.: On the development of insulin sensitivity by mouse mammary gland in vitro. Develop. Biol. Suppl. 4: 101-113, 1970.

Topper, Y. J. and Oka, T.: Steroids and the development of mammary epithelial cells. In Rabin, B. R. and Freedman, R. B. (Eds.): Effects of Drugs on Cellular Control Mechanisms. London, Macmillan, 1971, pp. 131-150.

Topper, Y. J., Voytovich, A. E. and Owens, I. S.: Postmitotic actions of prolactin and insulin on mammary gland extracts. In Hamburgh, M. and Barrington, E. J. W. (Eds.): Hormones in Development. New York, Appleton-Century-Crofts, 1971, pp. 61-65.

Topper, Y. J. and Green, M. R.: Some effects of prolactin, insulin and hydrocortisone on RNA synthesis by mouse mammary gland in vitro. In Falconer, I. R. (Ed.): Lactation. London, Butterworths, 1971, pp. 239-248.

Oka, T. and Topper, Y. J.: Hormone-dependent accumulation of rough endoplasmic reticulum in mouse mammary epithelial cells in vitro. J. Biol. Chem. 246: 7701-7707, 1971.

Oka, T. and Topper, Y. J.: Insulin-sepharose and the dynamics of insulin action. Proc. Nat. Acad. Sci. U.S. 68: 2066-2068, 1971.

Topper, Y. J. and Owens, I. S.: Development of the lactose synthetase system. Proc. Symp. on the Biochem. of Glycosidic Linkage, Bariloche, Argentina. (In press).

Serial No. NIAMD-LBM-8
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Biosynthesis of Inositol in the Mammal

Previous Serial Number: NIAMD-LBM-8

Principal Investigator: Dr. Frank Eisenberg, Jr.

Other Investigator: Dr. Conrad Chen

Cooperating Units: None

Man Years:

Total: 2
Professional: 2
Other: 0

Project Description:

Objectives - The mechanism of action of enzymes catalyzing internal oxidoreductions is of continuing and general interest. The NAD-dependent cyclase which catalyzes the cyclization of glucose 6-phosphate to inositol 1-phosphate is one of this class and, characteristically, is resistant to elucidation of its route of hydrogen transfer. Although isotope effects with deuterium labeled substrates on the rate of cyclization suggest that activation of glucose 6-phosphate, preliminary to cyclization, occurs at C5, no direct evidence for the intermediate existence of 5-ketoglucose 6-phosphate and inosose 1-phosphate has been adduced. Further attacks on this problem are in progress.

Methods Employed - Although NADH is a presumed intermediate in NAD-dependent oxidoreductions, attempts to demonstrate incorporation of ^{3}H into inositol 1-phosphate from exogenous reduced tritiated pyridine nucleotide have failed. An alternative approach is the use of oxidized tritiated pyridine nucleotide and examination of the product, inositol 1-phosphate, for tritium. Since NAD is obligatory there can be no question of the involvement of this species in the enzymatic reaction. But transfer of ^{3}H to the product is possible only if the stereospecificity of dehydrogenation of substrate and hydrogenation of intermediate are opposite-sided with respect to the nicotinamide ring. Failure to observe ^{3}H in the product would thus be inconclusive, due either to same-sided stereospecificity or a role for NAD not involving H transfer.

Further application of butaneboronic acid to the gas chromatography of carbohydrates has been investigated.

Experiments were performed to confirm the finding of Sherman et al. (Biochemistry 10: 3491, 1971) of the existence of a pathway in rat testis for the biosynthesis of neoinositol.

Major Findings - No isotope incorporation into inositol was observed when glucose 6-phosphate and NAD-4-^{3}H were incubated with purified testicular cyclase. As indicated above a negative result is inconclusive in defining the role of the coenzyme.

The butaneboronic acid derivatization reaction for the gas chromatography of carbohydrates has been extended to amino sugars and uronic acids. Thus neutral, basic, and acidic carbohydrates can be analyzed by this method, which is now being applied to the study of the hydrolysis of mucopolysaccharides by the Hunter and Hurler factors of Neufeld. The role of the Hurler factor as an iduronidase has been confirmed by this method.

The formation of neoinositol, an isomer of myoinositol, from mannose 6-phosphate by a soluble rat testis enzyme has confirmed the original observation of Sherman. The difficulty experienced by Sherman in separating neoinositol from mannose as the trimethylsilyl derivatives by gas chromatography has been overcome in this study by the use of the butaneboronates.

Significance to Bio-medical Research and the Program of the Institute - The source of the enzyme system used in these studies is rat testis. Since little is known about the biochemistry of this tissue delineation of a new pathway of carbohydrate metabolism is in the interest of metabolic research.

An improved analytical procedure for carbohydrate will accelerate progress in the elucidation of mucopolysaccharide and glycoprotein structure, areas of great current interest.

Proposed Course of Project - A further attempt at trapping an intermediate in the cyclization of glucose 6-phosphate will involve treatment of an actively cyclizing system with $NaB^{3}H_4$ and examination of the reaction products. If 5-ketoglucose 6-phosphate and inosose 1-phosphate are intermediates then owing to the stereononspecificity of borohydride reduction labeled iditol and scyllitol, respectively, should be detected in addition to labeled glucose and myoinositol. Separations will be effected by the gas chromatography of the butaneboronates.

Honors and Awards: None

Publications:

Eisenberg, F. Jr.: Cyclic butaneboronic acid esters: novel derivatives for the rapid separation of carbohydrates by gas-liquid chromatography. Carbohydrate Research 19: 135-138, 1971.

Eisenberg, F. Jr.: Inositol 1-phosphate. In Bergmeyer, H. U. (Ed.): Methods of Enzymatic Analysis (ed. 3). New York, Academic Press, (in press).

Eisenberg, F. Jr.: Gas chromatography of phosphorus compounds. In Kolthoff, I. M. and Elving, P. J.: Phosphorus Analysis. New York, Interscience, (in press).

Eisenberg, F. Jr.: Gas-liquid chromatography of carbohydrates as butaneboronic acid esters. In Ginsburg, V. (ed.): Methods in Enzymology. New York, Academic Press, (in press).

Serial No. NIAMD-LBM-9
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Enzymatic Reduction of Disulfide Linkages in Mammalian Cells.

Previous Serial Number: NIAMD-LBM-9

Principal Investigator: Dr. Frank Tietze

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 1
Professional: 1
Others: 0

Project Description:

Objectives - To study the subcellular distribution, specific functions, and mechanisms of action of several enzyme activities associated with the catalysis of thiol-disulfide interchange reactions in mammalian tissues.

Methods Employed - Rat liver or other mammalian tissues are fractionated into their subcellular particulate and soluble components by differential centrifugation. The components thus isolated are examined for their ability to catalyze the following interaction between glutathione (GSH) and disulfide-containing substrates (RSSR):

$$2GSH + RSSR \rightleftharpoons GSSG + 2RSH$$

The progress of this reaction is followed continuously in the spectrophotometer by measurement of the amount of GSSG formed.

Major Findings - The current study of the subcellular distribution of enzymes catalyzing thiol-disulfide exchange reactions (thiol-disulfide transhydrogenases) in rat liver has yielded the following information: 1) The post-microsomal (soluble) fraction contains a single activity associated with the catalysis of interchange reactions between glutathione (GSH) and substrates. 2) The microsomal fraction possesses two additional transhydrogenase activities which are distinct from the supernatant enzyme. While both of these microsomal enzymes possess GSH-insulin transhydrogenase activity, only one is capable of catalyzing interchange reactions between GSH and small

disulfides. 3) The mitochondrial and lysosomal fractions appear to be inactive with respect to the above enzyme activities.

Significance to Bio-medical Research and the Program of the Institute - Variations in thiol-disulfide ratios have been found to accompany a variety of physiological processes associated with cellular proliferation such as liver regeneration and other forms of wound healing, intra-nuclear events during mitosis, and budding phenomena in certain microorganisms. It is possible that a more precisely defined relationship between these processes may result from a study of enzymically catalyzed thiol-disulfide interchanges in model systems of the type employed in this investigation.

Proposed Course of Project - Studies will continue on the subcellular distribution, mode of action, and physiological significance of several distinct enzymes in rat liver found to catalyze sulfhydryl-disulfide interchange reactions. The fractionation procedures described above will be extended to include a number of additional subcellular components (e.g., plasma membrane, nucleus) not as yet studied.

Honors and Awards: None

Publications:

Tietze, F., Bradley, K. H. and Schulman, J. D.: Enzymic reduction of cystine by subcellular fractions of leukocytes from normal and cystinotic individuals. Pediatric Research (in press).

Tietze, F.: Enzymic reduction of disulfides. In Schulman, J. D. (Ed.): Cystinosis. Washington, D. C., U. S. Government Printing Office (in press).

Serial No. NIAMD-LBM-10
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Biochemical Lesions in the Genetic Mucopolysaccharidoses

Previous Serial Number: NIAMD-LBM-10

Principal Investigator: Dr. Elizabeth Neufeld

Other Investigators: Dr. Gideon Bach, Dr. Pamela Roddy, Mrs. Clara Hall and Mr. James Scott

Cooperating Units: Laboratory of Nutrition and Endocrinology, NIAMD

Man Years:

Total: 5.5
Professional: 4
Others: 1.5

Project Description:

Objectives - To determine the biochemical defect of the genetic disorders of mucopolysaccharide metabolism, and to use the findings for medical assistance to the patients and their families.

Methods Employed - Fibroblasts derived from skin of normal individuals and affected patients are grown in tissue culture, and used to study the mucopolysaccharide metabolism in the genetic disorders as well as to assay the corrective factors. The factors, in turn, are obtained from normal human urine and purified by conventional biochemical techniques. Radioactive mucopolysaccharides are prepared by administration of labeled precursors to fibroblasts and purification by the gentlest methods available.

Major Findings - 1. The factor deficient in Sanfilippo fibroblasts of the A subtype has been shown to degrade the heparan sulfate that accumulates in those fibroblasts. The Sanfilippo A factor is most probably a heparan sulfate N-sulfatase.

2. The Hurler and Scheie syndromes had been previously shown to have a deficiency of, and to be correctible by the same protein - designated "Hurler factor". This factor has now been shown to be α-L-iduronidase; consequently, the Hurler and Scheie syndromes are α-L-iduronidase deficiency diseases.

3. Normal fibroblasts in culture show an impairment in mucopolysaccharide degradation when the pH of medium is raised to 7.4 or higher. At pH 8, normal cells are not distinguishable from those with a mucopolysaccharidosis. They grow, however, remarkably well at the higher pH.

Significance to Bio-medical Research and the Program of the Institute - The finding that fibroblasts from mucopolysaccharidosis patients are easily correctible has given hope that the patients may likewise improve with replacement therapy. Following the initial optimistic reports of a group at Baylor University, plasma infusions have been administered to a great many mucopolysaccharidosis patients in the United States and abroad with results ranging from remarkable improvement to negligible effects. The difficulty is that clinicians are presently administering plasma without any prior test of its potency with respect to corrective activity.

We hope that elucidation of the enzymatic defect in the Hurler syndrome will result in an improvement of the therapeutic approach. It will now be possible to pretest plasma and plasma fractions and monitor the changes in iduronidase level. Our attempt to scale up the purification of Hurler factor for a clinical trial has not been successful; however, it should be possible to develop new methods, based on affinity chromatography, by which large amounts of enzyme could be prepared economically.

The mucopolysaccharidoses are rare diseases and do not represent a serious public health problem. However, studies of enzyme replacement in this family of disorders may serve as a model for other genetic diseases, which in the aggregate involve a considerable patient population.

Proposed Course of Project - Several of the proposals presented last year are still being planned for the future: search for better sources and better methods of purification, with the ultimate goal of turning the factors from substances of research interest to drugs for patients; production of antibodies to search for cross-reactive mutants; if patient's cells have a cross-reactive but non-functional protein, attempt to activate it.

In addition, other avenues will be explored. The easy entry of α-L-iduronidase into fibroblasts, in contrast to the negligible uptake of I^{125}-albumin, suggests that certain chemical features on the iduronidase allow it to "home" in fibroblasts. The features responsible for recognition of proteins by fibroblasts and other cultured cells will be examined. The present limitation on enzyme replacement therapy is the tendency of injected lysosomal enzymes to end up in the liver, whereas the major clinical damage may be in the brain or heart muscle. Information acquired in culture of neuroblastoma cells (to use one example) may result in proteins tailored to go to the brain.

Honors and Awards: None

Publications:

Neufeld, E. F. and Cantz, M. J.: Corrective factors in the mucopolysaccharidoses. Trans. N. Y. Acad. Sci. 179: 580-587, 1971.

Barton, R. W. and Neufeld, E. F.: The Hurler corrective factor. J. Biol. Chem. 246: 7773-7779, 1971.

Neufeld, E. F.: The mucopolysaccharidoses. In Dorfman, A. (Ed.): Antenatal Diagnosis. Chicago, Univ. of Chicago Press, 1972, pp. 217-228.

Barton, R. W. and Neufeld, E. F.: A distinct biochemical deficit in the Maroteaux-Lamy syndrome (mucopolysaccharidosis VI). J. Pediatrics 80: 114-116, 1972.

Kresse, H. and Neufeld, E. F.: The Sanfilippo A corrective factor. J. Biol. Chem. 247: 217-228, 1972.

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Neufeld, E. F. and Barton, R. W.: Genetic disorders of mucopolysaccharide metabolism. In Gaull, G. E. (Ed.): Biology of Brain Dysfunction. New York, Plenum (in press).

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Serial No. NIAMD-LBM-11
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Enzyme Induction

Previous Serial Number: NIAMD-LBM-11

Principal Investigator: Dr. Irwin G. Leder

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 1
Professional: 1
Others: 0

Project Description:

Objectives - To study the inducible lac transport system in virus infected E. coli.

Methods Employed - An overproduction of lac permease is induced in E. coli which are unable to produce a normal cell membrane and which carry a defective lac transducing phage. Affinity chromatography is used to isolate and purify the lac permease protein from cell lysates.

Major Findings - A. The susceptibility of actively accumulated substrate pools to cold shock loss has been shown to be independent of the age of the culture and of the absolute osmolarity or buffer composition of the incubation or growth medium. A report concluding, in contrast to these studies, that cold shock causes negligible loss of accumulated thiomethyl galactoside, has been shown to have been based on studies with a strain of E. coli which is particularly resistant to cold shock. Efforts to adduce evidence that cold shock leads to the formation of transient hydrophyllic channels by demonstrating the non-catalytic entry of labeled substrates have been unrewarding.

B. The crystalline compound derived from heptaacetylmethylgalacturonate-thio-galactoside has been shown to specifically interfere with lac permease having no effect on amino acid accumulation.

Significance to Bio-medical Research and the Program of the Institute - Elucidation of the role, structure and integration of the lac carrier protein in the bacterial membrane will provide insight into the general properties of membranes and in particular how membranes interact with effector molecules in normal and in diseased states.

Proposed Course of Project - A modified Sepharose bearing a binding group analogous to galactosyl-thio-galactoside will be used to isolate the lac carrier protein by affinity chromatography from E. coli mutants which have been transduced by phage containing lac DNA and which are unable to synthesize membrane phospholipids.

Honors and Awards: None

Publications:

Leder, I. G.: Permeability of the Escherichia coli membrane to permease accumulated substrates. J. Bacteriol. (in press).

Serial No. NIAMD-LBM-12
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Synthesis and Secretion of Immunoglobulins

Previous Serial Number: NIAMD-LBM-12

Principal Investigator: Dr. Milton Kern

Other Investigators: Dr. Daniel H. Zimmerman and Dr. Thomas W. Mikulka

Cooperating Units: None

Man Years:

Total: 3
Professional: 2.5
Others: .5

Project Description:

Objectives - The purpose of this project is to study the nature of the intracellular mechanisms responsible for the synthesis and secretion of immunoglobulins and also to gain insight into the structure of that class of proteins known as secretory immunoglobulins.

Methods Employed - Immunoglobulin producing cells, incubated with ^{3}H-leucine, were lysed with deoxycholate in the presence of iodoacetamide to preclude further disulfide bond formation. The specifically precipitable immunoglobulin components derived from both intra- and extracellular fluids were analyzed. Some of the techniques used were sodium dodecyl sulfate - acrylamide gel electrophoresis, column chromatography and cyanogen bromide cleavage of peptides. Colostral IgA was purified from rabbit milk prior to its use for structural studies.

Major Findings - Incompletely synthesized heavy chains of immunoglobulin G were produced by rabbit lymph node cells incubated in the presence of 10^{-5} M puromycin. After cyanogen bromide treatment, such incomplete chains yielded peptides which overlapped with those derived from native heavy chains. Cells incubated with ^{3}H-puromycin produced radioactive materials which were precipitated with a serum specific for immunoglobulin G. Furthermore, reduced and alkylated ^{3}H-puromycin labeled immunoglobulins were smaller than heavy chains as judged by sodium dodecyl sulfate - polyacrylamide gel electrophoresis. Incomplete heavy chains were synthesized as well as secreted into the extracellular medium by lymph node cells incubated in the presence of puromycin.

Colostral immunoglobulin A was examined for possible subunits analogous to the immunoglobulin G-like subunit of immunoglobulin M. Reduction of such immunoglobulin A with dithiothreitol yielded three slower moving components on molecular sieve chromatography in the presence of physiological saline. One of the products was identified as light chain. Of the other two products, one appears to be composed of molecules having one each of heavy, light and secretory component chains. This structure best fits the data obtained from estimates of its molecular weight via column chromatography, from chain composition analysis and from the known molecular weight of its component polypeptide chains. Based on these same criteria, the remaining reduction product of IgA best fits a molecular structure composed of 3 heavy and 3 light chains.

Significance to Bio-medical Research and the Program of the Institute - The abnormal γ-globulins and their subunits that have been observed in serum and urine may be a consequence of aberrations in the secretory mechanism, as well as the synthetic mechanism. Studies from this laboratory have elucidated several new parameters that define synthesis and secretion of normal γ-globulin and provide a means for assessing the similarities and dissimilarities between a normal and abnormal cell. In addition, such parameters may also be useful in distinguishing whether the differentiation of lymphocytes to yield cells which produce and secrete γ-globulin develop the polypeptide synthesizing and secretory apparatus simultaneously or not.

It should be noted that the generation of recognizable incomplete polypeptide chains at appropriate concentrations of puromycin can be used for the study of other proteins. This procedure may have particular pertinence in the study of conjugated proteins such as glycoproteins and lipoproteins. In the case of glycoproteins, e.g., immunoglobulins, it may be useful as a means of generating sufficient quantities of "artificial nascent chains" to assess whether carbohydrate residues are acquired prior to completion of the peptide backbone. Furthermore, the generation of incomplete peptides in the presence of puromycin might be helpful in establishing the relationships between the large precursor protein molecules which apparently represent the primary viral gene products of cells infected with certain viruses and those much smaller proteins associated with the native virus particle.

Proposed Course of Project - Efforts will be directed toward determining whether some carbohydrate residues of immunoglobulin chains are acquired prior to completion of the peptide backbone. In addition, characterization of rabbit IgA from sources other than colostrum will be undertaken.

Honors and Awards: None

Publications:

Kern, M.: The role of carbohydrate in immunoglobulin secretion. In Jamieson, G. A. and Greenwalt, P. J. (Eds.): Glycoproteins of Blood Cells and Plasma. Philadelphia, J. B. Lippincott, 1971, pp. 190-203.

Sutherland, E. W. III, Zimmerman, D. H. and Kern, M.: Synthesis and secretion of gamma-globulin by lymph node cells: The acquisition of carbohydrate residues of immunoglobulin in relation to interchain disulfide bond formation. Proc. Nat. Acad. Sci. U. S. 69: 167-171, 1972.

Zimmerman, D. H. and Kern, M.: Synthesis and secretion of γ-globulin by lymph node cells. X. The generation of incompletely synthesized immunoglobulin heavy chains. J. Biol. Chem. (in press).

Serial No. NIAMD-LBM-13
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Particulate Enzymes of Carbohydrate Metabolism

Previous Serial Number: None

Principal Investigator: Dr. Marjorie R. Stetten

Other Investigators: Mr. Paul Goldsmith

Cooperating Units: None

Man Years:

Total: 2
Professional: 1
Other: 1

Project Description:

Objectives - To study the occurrence and properties of membrane bound enzymes of liver and kidney which catalyze the synthesis and hydrolysis of a variety of phosphorylated sugars and sugar alcohols.

Methods Employed - Microsomal membrane enzyme preparations from homogenates of organs of normal and abnormal rats are obtained by differential centrifugation methods and are activated by treatment with such reagents as deoxycholate, Triton X-100, digitonin or hydroxyl ion. Conventional chemical or enzyme assay methods, when applicable, are used for kinetic studies and quantitative measurements. When enzymatic methods are not available, radioactive P^{32} or C^{14} incorporation methods are employed. New phosphorylated products are isolated and characterized by chromatographic and chemical methods. Methods for the solubilization and purification of inorganic pyrophosphate-glucose phosphotransferase are being studied.

Major Findings - It has been found that the properties of glucose 6-phosphatase and related enzymes are different in rough and smooth endoplasmic reticular membranes. In membrane areas having attached ribosomes the enzyme is predominantly in an "active" configuration while that in the smooth membranes is in a less active or potential form.

A comparative kinetic study of the phosphorylation of the 5-carbon sugar alcohols by inorganic pyrophosphate and microsomal enzyme has been carried out. The four pentitols serve as acceptors for the phosphotransferase

activity from PP_i at varying rates: D-arabitol > ribitol > L-arabitol > xylitol. In livers of fasted and diabetic rats the levels of enzyme activity are greatly elevated over those found in normal fed animals. The product of the enzymatic phosphorylation of ribitol has been isolated and characterized as D-ribitol-5-phosphate.

Significance to Bio-medical Research and the Program of the Institute - The enzymes being studied are capable of using PP_i instead of ATP for the activation of sugars and sugar alcohols prior to their utilization by animal tissues. The quantitative levels of these membrane-bound enzymes have been found to be particularly susceptible in vivo to fasting and to a number of endocrine changes, such as result from diabetes, adrenalectomy, cortisone and insulin administration. Conditions of high glucose levels and increased enzyme activity suggest a physiological function of the PP_i-glucose-phosphotransferase for the synthesis of glucose 6-phosphate in the diabetic animal.

It is hoped that these studies may contribute to a better understanding of normal and abnormal carbohydrate metabolism.

Proposed Course of Project - Studies of the solubilization and purification of the membrane enzymes will be continued in an attempt to answer the question of whether phospholipid is required for the activity or for the configurational stability of the enzymes. Acceptor specificity studies will be extended to include the possible enzymatic synthesis of sugar diphosphates.

Honors and Awards: None

Publications:

Stetten, M. R. and Ghosh, S. B.: Different properties of glucose-6-phosphatase and related enzymes in rough and smooth endoplasmic reticular membranes. Biochim. Biophys. Acta 233: 163-175, 1971.

Stetten, M. R. and Kehoe, J. J.: Synthesis of D-ribitol 5-phosphate by an inorganic pyrophosphate-ribitol phosphotransferase activity of microsomal glucose-6-phosphatase. Biochim. Biophys. Acta 250: 501-513, 1971.

SECTION ON BIOCHEMICAL MECHANISMS

Stereopopulation Control in Simulation of Peptide Bond Formation

Studies have continued on the development of models for enzyme-catalyzed peptide bond formation and transpeptidation. Starting with N-o-aminophenyl peptides, substituents have been added to the phenyl ring which produce severe conformational restriction. By virtue of its steric and hydrogen bonding effects, the o-nitro group is so effective that lactamization and transpeptidation occur rapidly over the pH range -2 to 12, at rates too fast to measure by standard techniques. These models demonstrate that enzymatic peptide bond formation does not necessarily require a means of carboxyl activation and can be driven by entropic freezing alone (L. A. Cohen and K. L. Kirk).

Stereopopulation Control in Simulation of Enzyme Catalysis

Based on the hypothesis that an enzyme active site remolds the substrate into a geometry and electronic state approaching that of the transition state, model compounds have now been prepared in which the activation energy for reaction is so close to zero that the ground state cannot be isolated. Except for general acid-base functions, these models (3,3,4-trimethyl and 3,3,4,7-tetramethylphthalide) contain most of the factors believed to be significant in enzyme catalysis (L. A. Cohen and P. S. Hillery).

Stereopopulation Control in Reduction of the Electrostatic Gap

Analogs of histidine have been synthesized in which the carboxyl group is held so close to the imidazole ring that solvation of the zwitterionic sites becomes impossible. Although such molecules show the spectroscopic properties of ionic species, pK_a values are vastly distorted and physical properties (melting point, volatility, solubility) are those of nonionic molecules. The behavior of these compounds demonstrates how the rigid matrix of a protein can, by restricting solvation, displace the pK_a values of ionizable groups to regions many units away from the normal ones (L. A. Cohen, Y. Kikugawa, H. Yeh and J. S. Cohen).

Stereopopulation Control in the Formation of High Energy Bonds

Analogs of imidazolepropionic and butyric acid have been synthesized in which the side chain is held rigidly so close to the imidazole ring that spontaneous cyclization to acylimidazole occurs. Since the free energy of hydrolysis of acylimidazoles is 6-8 kcal per mol greater than that of normal amides, these models demonstrate that entropic freezing alone can be utilized, at the enzymatic site, to generate high energy bonds (L. A. Cohen and Y. Kikugawa).

Stereopopulation Control in Electronic Activation of a Substrate

It has been proposed that lysozyme activates its substrate by deforming the normal chair conformation of a cyclic sugar to a half chair, thus increasing the electronic stability of a carbonium-ion intermediate. Model structures have now been synthesized in which slight distortion of the pyranose ring increases rates of hydrolysis 3000-4000 fold. Mechanistic studies show this effect to be due to enhanced conformational stabilization of carbonium-ion intermediates (L. A. Cohen and R. T. Borchardt).

Fluoro and Amino Analogs of Histamine and Histidine

Diazonium coupling of N-acetylhistamine and N-acetylhistidine produces complex mixtures of 2-, 4-, and 2,4-bis-phenylazo derivatives. Careful chromatography has permitted the isolation of the pure 2-phenylazo derivatives. Reduction and hydrolysis of these compounds provides, for the first time, 2-aminohistamine and 2-aminohistidine. Photochemical fluorination of the latter compounds (by our recently discovered method) leads to the 2-fluoro-analogs.

These compounds are being tested as antihistaminic agents. The isomeric 2-fluoro and 4-fluorohistidine are being substituted for histidine (by total synthesis) in polypeptide hormones and in ribonuclease-S-peptide. 4-Fluorohistidine has been found to be a substrate for histidine deaminase, D-amino acid oxidase and histidine decarboxylase. The N-acetyl derivatives are also hydrolyzed by hog kidney acylase (L. A. Cohen and K. L. Kirk).

Selective Modification by Protein-Polymer Interaction

An insoluble polymer has been synthesized containing N-iodosuccinimide units. Kinetic studies demonstrate that the polymer is capable of iodinating tyrosine in proteins, at low pH, by a direct mechanism requiring full contact between polymer and protein. The ability to utilize this phenomenon to iodinate selectively only the very highly exposed tyrosine residues is being explored. Polymer containing radioactive iodine has been prepared and is being tested as a reagent for the selective radiolabelling of tryosine in erythrocyte membranes. Further possibilities include labelling of the binding sites of immunoglobulins and identification of the binding sites of polypeptide hormones to membranes (L. A. Cohen and R. Jerussi).

Electronic Properties and Conformation of Phenolic Esters

Correlation of carbonyl frequencies and of ultraviolet spectra of phenolic esters with σ^{o} shows the absence of any through resonance or coupling of the phenolic oxygen to the benzene ring. This effect is due to preferential resonance of the oxygen atom with the carbonyl. About forty studies of interaction of phenolic esters with nucleophiles have been described in the literature; in none of these cases was a Hammett correlation successful. The data have now been reanalyzed by computer correlation with σ^{o} and found to be totally successful. An additional and unexpected result is the observation that while tetrahedral formation is rate limiting in intermolecular reactions, tetrahedral breakdown is rate limiting for intramolecular cases. These results

will require a reevaluation of data and conclusions on the interaction of hydrolytic enzymes with phenolic esters and anilides (L. A. Cohen).

SECTION ON CARBOHYDRATES

Aldonic Acids and 2-Acetamido-2-deoxyaldonic Acids

Although they play manifold roles in biochemical systems, the aldonic acids often present marked difficulties for the investigator. The isolation of these substances is complicated by the fact that they normally exist in aqueous solution as a complex equilibrium mixture consisting of the free aldonic acid, the corresponding 1,4-lactone and the 1,5-lactone. We have found that the isolation and characterization of aldonic acids is markedly facilitated through the preparation of their salts with dicyclohexylamine. Such salts may be prepared from metal aldonates, from aldono-1,4-lactones and from aldono-1,5-lactones. The first known dicyclohexylammonium aldonate, dicyclohexylammonium 2-acetamido-2-deoxy-D-gluconate, was mentioned in a previous report, and it was noted that the same substance may be obtained through the action of dicyclohexylamine on 2-acetamido-2-deoxy-D-mannono-1,4-lactone. The preparation of the salt has now been improved and its configuration has been confirmed through conversion to 2-acetamido-2-deoxy-D-glucose. Furthermore, the configuration of the presumed 2-acetamido-2-deoxy-D-mannono-1,4-lactone has been reconfirmed through its reduction to 2-acetamido-2-deoxy-D-mannitol. It is thus now firmly established that aqueous dicyclohexylamine may cause the epimerization of 2-acetamido-2-deoxyaldonic acid derivatives although the exact point at which the epimerization takes place remains to be ascertained. We have conclusively shown that the dicyclohexylammonium salt which may be isolated subsequent to the bromine-water oxidation of 2-acetamido-2-deoxy-D-galactose is a 2-acetamido-2-deoxy-D-galactonate.

Some 2-acetamido-2-deoxy-D-aldono-1,4-lactones, notably those of the D-*manno* and D-*galacto* series resist reduction with buffered sodium amalgam -- a reagent which has been widely used for the reduction of non-nitrogenous aldonolactones. Sodium borohydride, however, readily reduces 2-acetamido-2-deoxy-D-mannono-1,4-lactone although more conveniently to the glycitol rather than to the aldose stage. 2-Acetamido-2-deoxy-D-glucitol is readily distinguished from its D-*manno* isomer inasmuch as the optical rotations of these glycitols are markedly enhanced when taken in acidified molybdate solution (E. Zissis, H. W. Diehl, and H. G. Fletcher).

Unsaturated Aminosugars

In earlier work, substances such as 3,4,6-tri-*O*-acetyl-2-(*N*-acetylacetamido)-1,2-dideoxy-D-*arabino*-hex-1-enopyranose were shown to be susceptible to attack by nucleophiles in the presence of a trace of strong acid. Further study of this type of reaction has shown that such unsaturated aminosugar derivatives undergo stereospecific rearrangments when heated in the presence of an acid catalyst alone. This advance has made available a variety of 2-amino-2,3-dideoxyhex-2-enopyranose derivatives and these, in turn, have been used as substrates in a study of the behavior of unsaturated aminosugar derivatives in the presence of hydrogen and of a

palladium catalyst. While unsaturated acetamidosugars are readily reduced to saturated acetamidosugars, the presence of a di-N-acetylamino group, attached to a carbon atom bearing a double bond, appears to inhibit totally the catalytic reduction of the double bond. Other reactions can, however, take place, notably hydrogenolytic cleavages and allylic shifts. These are under investigation (N. Pravdić, B. Zidovec and H. G. Fletcher, Jr).

The Synthesis of Ribonucleosides

2,3,5-Tri-O-benzoyl-1-O-(p-nitrobenzoyl)-β-ribofuranose has been prepared and characterized. The utility of the substance for the synthesis of β-D-ribofuranosides has been demonstrated and details for the synthesis of the intermediate in multi-kilogram lots have been worked out (R. K. Ness, K. W. Freer, and H. G. Fletcher, Jr.).

Immunochemistry

Two immunoglobulins from plasmacytomas were found to precipitate with arabogalactans. Immuno determinants were elucidated by studying hapten inhibition. Pure homogeneous immunoglobulins have been isolated by affinity chromatography, employing a sepharose support, coupled to haptens. These globulins are being investigated at present (C.P.J. Glaudemans and M. Potter, NCI)

SECTION ON MEDICINAL CHEMISTRY

Computers in Drug Design

The biological activity of the N-substituted morphinan analgesics was correlated, through computerized regression analyses (IBM 360/370 via Wylbur), with the substituents' electronic and steric parameters. Correlation of the COMT catalyzed O-methylation of neutral catechol substrates vs their chemical structure, again by computerized regression analyses, was found with the steric effect of the substituent in the catechol and the molecule's transport to the enzyme surface. The development of a combination of computer programs for the direct and immediate operator interaction with a graphics computer system (PDP 10 via SOS) allowed the rapid, precise determination of nmr chemical shifts and coupling constants of complex drug molecules. What ordinarily might have been accomplished in weeks can now be achieved in hours, with this combined program (A. E. Jacobson).

Transformations and NMR Studies in the Codeine Series

Reaction of Codeinone with excess dimethyloxosulfonium methylide gave instead of the expected exo-cyclopropyl ketone a small yield of a novel codeinone derivative containing the spiro oxirane system at the 6-position as shown by mass spectrometry and infrared and exhaustive nmr studies. Furthermore, reduction of this oxirane gave the new 6-methylisocodeine. Conformations of the oxirane, 6-methyl-codeine and 6-methylisocodeine were determined with a 100 MHz instrument and the Nuclear-Overhauser effect.

The spiro oxirane and 6-methylisocodeine are somewhat stronger analgesics than codeine (L. J. Sargent).

Research in Narcotics Abuse (Synthesis)

To provide new and improved agents for research in the fight against narcotic abuse, appropriate modifications (at the N-function) of ketobemidone (a powerful analgesic with high addiction liability), of (±)-5-m-hydroxyphenyl-2-methylmorphan (a strong analgesic with medium potential for abuse) and the (-)-isomer of the latter (a mixed agonist-antagonist with low abuse potential) have been made. These three models contain the phenolic hydroxyl meta to a quaternary-carbon linkage (a presumed prerequisite for agonist or antagonist effect in this type of molecule) but not two or more contiguous fused, carbocyclic rings also postulated to be a necessary structural requisite for antagonistic property. Groups substituted on nitrogen for methyl in the ketobemidone series were ethyl through heptyl, cyclopropylmethyl, allyl, and propyl. The last three groups only, replaced methyl in the two phenylmorphan series. Analgesic activity (in mice, hot-plate and Nilsen) ranged from three times the potency of morphine to none. These compounds are being assessed for antagonistic property and abuse potential in monkeys. An interesting "offshoot" of this research was the finding that cyclopropylcarbonyl chloride (but not benzoyl, acrylyl, propionyl chlorides) will expel an N-methyl group to give the N-cyclopropyl carbonyl analog in 80% yield. If such an amide can be readily hydrolyzed, an improved N-demethylation procedure will be provided (T. Oh-ishi, H. Ong and E. L. May).

A Copper-Specific Binding Agent

A direct method of glucuronidation of methanol, phenol and phenethanol has been developed. The phenethyl-β-glucuronide so prepared (in 52% yield) was shown to complex with Cu but not with Na, K, Ca, Mg, Fe, Co, Mn or Zn at pH 7.8. This suggests therapy potential for diseases induced by disorders of copper metabolism (J. G. Murphy).

Evaluation of New Agents for Research in Narcotic Abuse

In the testing of 45 new compounds from the Section on Medicinal Chemistry, the pharmaceutical industry and universities, several candidates for further study were uncovered. Particularly notable are two antagonists that have emerged as possible substitutes for naloxone and cyclazocine now being studied as deterrents to heroin abuse. One is the longest-acting antagonist yet examined; it has an unusual structure for this type of action. The Nilsen test, installed about two years ago, has been refined to the point where it is not only more predictive than the hot-plate method for the mixed agonists-antagonists, but is more economical than and as accurate as the hot-plate for the pure agonists (E. L. May and N. B. Eddy).

Photocyclizations

Photolysis of N-chloroacetyl-N-methyl-3-m-hydroxyphenylbutylamine according to a method developed by B. Witkop, et al., has given (p-ring closure) 30-35% yield of an 8-membered lactam which on hydride reduction afforded 3,6-dimethyl-1,2,3,4,5,6-hexahydro-8-hydroxy-3-benzazocine, a homolog (lacking the 5,9-methano bridge) of the 6,7-benzomorphans. In the photocyclization reaction, no ortho-ring closure was observed, and if a corex rather than a vycar filter was used, no ring closure (only conversion of chloroacetyl to acetyl) took place (H. Ong).

Instrumentation

Further research on the automatic viscometer developed in this section indicates that the automatic-fill mechanism need not be thermostatted and that for practical application to biomedical problems, one should have a totally sealed system of 1 ml capacity (T. D. Perrine).

Colombian Bark Extract

An aqueous alcoholic extract of bark from Colombia (J. W. Daly), claimed to be beneficial in arthritic conditions, gave a shiny brown solid (after evaporation of solvents) with an initial mass peak of 207, changing gradually on storage to a high of 305. Elemental analysis showed 48% C, 4% H and no nitrogen. It was essentially inactive as an analgesic in mice (by oral or subcutaneous administration) but caused 49% inhibition of carrageenan-induced, foot-pad edema in the mouse at a dose of 500 mg/kg. Attempts are underway to isolate pure components of this extract (J. Ager).

Asparagine Synthetase and Tumor Inhibitors

1,4-Bis-N-piperidino-2-aminobutane and the 2-methylamino analog derived from L-aspartic acid were non-cytotoxic (at 100 μg/ml) to two murine leukemia cell lines (R. Adamson, NCI) in contrast to the corresponding 1,4-phenethylamino congeners which gave 70-95% inhibition of cell growth at 10-100 μg/ml. The two piperidino compounds also failed to inhibit asparagine synthetase (D. Cooney, NCI). Corresponding members of the D-series are yet to be tested.

Optical rotations on various members of the L- and D-series indicate that no racemization occurred during the carbodiimide-induced amide preparations or during hydride reductions (in aprotic solvents). However, some racemization seems to have taken place during the hydrogenolysis ($Pd-BaSO_4$, methanol) reactions.

Some amino hydrazides of D-aspartic acid were prepared from N-carbobenzoxy-D-aspartic acid and hydrogenolysis (Pd-C). These will be assessed for tumor and asparagine synthetase inhibition (J. H. Ager and E. L. May).

Studies on Batrachotoxin, Pumiliotoxin, Histrionicotoxin A and Other Physiologically Active Compounds from Amphibian Skins

Investigation of the unique pharmacological properties of batrachotoxin and various analogs has continued. Batrachotoxin appears to increase membrane permeability to sodium ions by interaction with certain membranal proteins. The effect of the drug can be blocked by mild pretreatment of the membrane with sulfhydryl reagents. The effect of batrachotoxin on spontaneous neuromuscular transmission in Ca^{++}-free media provides evidence for the presence of a mechanism, coupling membrane depolarization to transmitter release, which exhibits a remarkable Q_{10} of >25.

The spiropiperidine alkaloids such as histrionicctoxin isolated from frogs of the genus Dendrobates exhibit a spectrum of interesting pharmacological properties. They first cause a rapid potentiation of the twitch evoked by either direct or indirect stimulation of neuromuscular preparations. They then block the effect of acetylcholine on the muscle endplate receptor. They antagonize the increase in potassium ion conductance associated with action potentials in muscle and nerve, but have no effect on potassium conductance of the resting membrane. After washing to restore the responsiveness of the muscle fiber to acetylcholine, another relatively irreversible effect of such toxins is revealed. Under these conditions they appear to markedly slow the rate of restoration of responsiveness of the acetylcholine receptor after one stimulation. The molecular basis for these properties is under investigation. Slight structural modifications in the toxins result in remarkable shifts in the relative effects on the various components of the cholinergic system. Compounds with almost pure receptor antagonist activity and those with almost pure effects on potassium ion conductances have been revealed.

Comparative chemical taxonomy and pharmacological taxonomy with frogs of this family reveal that batrachotoxin-like compounds and a marked insensitivity to the depolarizing action of batrachotoxin on electrogenic membranes are associated with certain frogs of the genus Phyllobates, while histrionicotoxin-like compounds and a marked insensitivity of the acetylcholine receptor to histrionicotoxin are typically associated with frogs of the genus Dendrobates.

Synthetic Program: Batrachotoxinin A was synthesized in a sequence involving 43 steps by H. Wehrli in the laboratory of Prof. O. Jeger, ETH, Zurich, in March 1972. The conversion to Batrachotoxin is known and has produced the first fully synthetic frog venom.

Pumiliotoxin C is rapidly approaching completion. The synthesis is in progress in the laboratory of Prof. G. Habermehl at the Technische Hochschule in Darmstadt.

Histrionicotoxin's synthesis is well under way in a 14-step sequence in the laboratory of Prof. E. J. Corey, Harvard University.

The exceptionally high toxicity of batrachotoxin, third among known toxins, its action on nerve preparations and its cardiotoxin properties gave the elucidation of its structure and correlations of structure with activity a high priority in biomedical research. Other compounds from frog extracts also have physiological activities which warrant their structural and pharmacological investigation. Examples of such compounds occurring in frogs and toads include the pumiliotoxins, histrionicotoxins, samandarine, the bufogenins, the catecholamines, indolealkylamines and histamines, and recently hypotensive polypeptides such as bradykinin and physalaemin. Because of its structural similarity to bufotenine and lysergic acid diethylamide, a thorough investigation of the properties of dihydrobufotenine is relevant to fundamental aspects of neurochemistry. The novel structure of histrionicotoxin related as it is to that of other alkaloids active in cholinergic mechanisms, warrants a thorough study both of its chemistry and its pharmacology (B. Witkop, J. Daly and D. Johnson).

Photochemistry of Pharmacodynamic Amines

Irradiation of N-chloroacetyltyrosine in 10% aqueous ethanol gave the ethyl acetate-soluble 7-hydroxy-1,2,4,5-tetrahydro-3H-3-benzazepin-2-one, and two water-soluble dimers in yields of 40 and 12%, respectively. X-ray analyses of single crystals by the symbolic addition procedure established the structure of the 1st dimer as decahydro-7,14a,7a,14-ethanediylidenenaphtho[1,8-de:4,5-d'e']bisazocine-4,6,11,13-(1H,7H,8H,14H)-tetrone, and the second dimer as 4,11-diacetyl-dodecahydro-7H-1,7,8a-ethanyylidene-8,14-methanocyclopropa[1,6]benzo[1,2-d:4,3-d']bisazocine-3,12,15,17-(4H,9H)-tetrone. The first dimer is converted to the second dimer on irradiation by a bond-switching process which is thought to be initiated by a Norrish-type I cleavage. Mono- and dimethyl homologs of the two dimers were obtained by the irradiation of the corresponding N- or ring-methylated chloroacetyltyramines.

On the basis of a study on solvent effects in the photolysis of N-chloroacetylmescaline a dualistic mechanism, intramolecular electron transfer in water and protic solvents versus intramolecular energy transfer in most organic solvents is proposed.

There is no oxygen effect in the formation of the novel ten-membered lactams from N-chloroacetamides of 3-methoxy- and 3,5-dimethoxyphenethylamines suggestive of a mechanism which consists in hydrogen abstraction and intramolecular recombination within the solvent cage (B. Witkop).

The Induction of Interferon by Synthetic Polynucleotides

In order to define molecular parameters which are important in determining the efficacy of a polynucleotide as an inducer of interferon, a number of compounds (e.g., poly 2'-azido-d U and poly 3-methyl U) were synthesized, characterized, and evaluated as inducers. The results of these studies have indicated a number of important properties a polynucleotide should possess. Also, the enzyme in human serum which degrades these polynucleotides appears to have a different substrate specificity from bovine pancreatic ribonuclease, a model enzyme previously used in correlating interferon induction (P. Torrence and J. A. Waters).

Synthesis of Antitumor and Antiviral Nucleosides

A novel, two-step synthesis of 5-mercaptouridine and 5-mercapto-2'-deoxyuridine was achieved by addition of thiocyanogen chloride to the nucleoside to form the 5-thiocyanato derivatives, which were subsequently reduced to the mercapto analogs using sodium dithionite or dithiothreitol. The compounds will be tested for their cytotoxicity and antiviral activities (T. Nagamachi, P. Torrence and J. A. Waters).

Physical Properties of Modified Polynucleotides

Poly 2'-azido-2'-deoxyuridylic acid (poly U_Z) was synthesized and its physical properties examined in order to determine the significance of the 2'-hydroxyl group in RNA. These studies have shown for the first time that a 2'-hydroxyl or 2'-oxygen is not necessary for stability of the secondary structure in a polyribonucleotide (P. Torrence and J. A. Waters).

Fluorescent Derivatives of Strophanthidin as Inhibitors of Sodium-Potassium-Dependent Adenosine Diphosphatase

Strophanthidin derivatives were tested as inhibitors of Na^+/K^+-dependent ATPase. The strophanthidin 3-O-esters showed very strong Na^+/K^+-dependent ATPase inhibitory activities (appreciably stronger than ouabain). Two of the compounds, the 3-salicylate and the 19-salicylhydrazone, showed strong fluorescent properties which may prove useful in the study of enzyme-substrate interactions of cardiac glycosides (J. A. Waters, F. Oesch).

Novel Pyrrole Esters of Alkaloids and Steroids

Since the powerful and selective ion transport agent batrachotoxin possesses a uniquely substituted pyrrole-3-carboxylic acid group, other esters of pharmacologically active steroids and alkaloids have now been prepared as potentially useful tools for the neurophysiological laboratory. Esterification of codeine, scopoline, jervine, methyl reserpate, ephedrine, and cholesterol with the pyrrole carboxylic acid has been achieved *via* a trifluoroacetic-pyrrole carboxylic anhydride intermediate. The reaction conditions are favorable for the acid-labile pyrrole acid, as well as the alkaloid portion of the esters. The compounds will be screened for various pharmacological activities (J. A. Waters).

The Reaction of Indoles with Diazotized o-Methoxy-p-nitroaniline [Fast Red-B]

As models for the reaction of tryptophan in proteins with the histochemical reagent, Fast Red-B, we have studied N-acetyltryptophan ethyl ester, one monomethyl- and two dimethyl-indoles. Skatole gives two products whose ratio is dependent upon pH. At pH 3.0, a 2-azo product predominates while at pH 7.0, the major product is a pyrrole-ring cleaved aldehyde. 1,2-Dimethylindole affords a 3-azo product while 2,3-dimethylindole gives, at either pH 3 or 7, the o-methoxy-p-nitrophenylhydrazone of 2-formylskatole. N-Acetyl-L-tryptophan ethyl ester, at pH 7.0, affords a 3a-azo substituted pyrrolo[2,3-b] intermediate [a "cyclo-tryptophan"] accompanied by minor amounts of 2-azo and

pyrrole-ring cleaved products. At pH 3.0, two bis-labelled products arise as well as the 2-azo product and surprisingly, a 5-azo substituted tryptophan ester. The bis-labelled compounds were shown to be intermediates to this 5-azo material and to be derived from the cyclotryptophan. This unprecedented electrophilic substitution reaction makes possible the direct functionalization of the biologically important 5-position of tryptophan with such substituents as amino, hydroxyl and fluoro groups (T. F. Spande, LC, and George Glenner, LP).

Synthesis of Anhydrogliotoxin Analogs

The Leuchs' addition of suitably functionalized α-halo α-amino acid chlorides to indolenine-2-carboxylic acid esters should provide reactive intermediates which may be reacted with disulfide ion or sulfur-containing nucleophiles to generate disulfide intermediates capable of undergoing an intramolecular ester-aminolysis reaction to afford dithio bridged dioxopiperazines. A number of model reactions testing this approach have been explored and indicate promise. The same scheme should be applicable to the synthesis of sporidesmin analogs using a pyrrolo[2,3-b]indolenine ester. Activities against bacteria, mycobacteria, fungi and viruses might be expected from gliotoxin or sporidesmin analogs (H. C. J. Ottenheym and T. F. Spande).

SECTION ON MICROANALYTICAL SERVICES AND INSTRUMENTATION

Service Function and Instrumentation

A total of some 12,000 analyses were performed on a service basis. These include: Carbon 1719, hydrogen 1719, nitrogen 4111 (Kjeldahl, Dumas, and Nessler), phosphorus 308, sulfur 114, halogens 198 and a variety of metals and miscellaneous functional groups. Instrumental analysis was also performed by infrared, nuclear magnetic resonance and mass spectra accounting for an additional 1800 spectral analyses. The NMR service has been expanded by the acquisition of a Varian HA-100 spectrometer and over 400 spectra have been analyzed and interpreted in the 6-month period of its operation (D. F. Johnson).

Synthesis of Tomatillidine from Solasodine

A former method of synthesizing tomatillidine from kryptogenin resulted in a loss of configuration at C_{25} in the final step, giving rise to doubt about the stereochemical interrelationship of several alkaloids previously investigated in this laboratory. At present, a 9-step synthesis, starting with solasodine, gave Δ^5-solacongestidine, with retention of configuration at C_{25}. Oxidation of this product in the production of tomatillidine is under study to resolve the question of isomerism at C_{25} (G. Kusano).

NMR Spectral Studies

A variety of research studies have been undertaken using the Varian HA-100 spectrometer. These investigations require highly speciallized interpretation and analysis rather than on a service basis. Projects in progress include: the stereochemical course of nucleophilic additions to arene oxides,

intramolecular interaction of (5-methylimidazole-4yl) dimethyl butyric acid and its analogs, ^{1}H and ^{19}F studies of ring-flourinated imidazole derivatives, magnetic anisotropy of a spiro oxirane ring and studies of mono- and di-methyl benzene oxide-oxepin valence tautomerism to name a few (H. J. Yeh).

Synthesis of Solaphyllidine

Solaphyllidine posseses several functional groups on a steroidal skeleton that suggests a possibility of pharmacological activity. Its synthesis, including side reaction products, therefore, provides data on the relationship between structure and biological activity of steroidal alkaloids. 4-Deoxo-Δ^5-solaphyllidine was oxidized to dihydro-Δ^5-solaphyllidine with SeO_2 and the latter was oxidized to Δ^5-solaphyllidine with active MnO_2. Selective reduction of the double bond at C_5 is being attempted, to convert Δ^5-solaphyllidine to solaphyllidine. Biological assay of certain reaction products is continuing (G. Kusano and Y. Sato).

Metabolism of C^{14}-Labelled Steroids by Adrenals from Pseudohermaphrodite Rats

A unique colony of rats has been under study in this laboratory in which a genetic defect, carried by normal females is passed on to half the genetic males. Half the genetic males are pseudohermaphrodites, having both male and female characteristics, although the testes are internal. It has been demonstrated by others that they have a defect of testosterone synthesis suggesting a general deficiency of the 17-α-hydroxylase system. Significant differences in adrenal metabolism of these pseudo rats was observed in this laboratory on incubation of C^{14}-progesterone. Unusual production of 4-androstene-3,17-dione, a direct precursor of testosterone, was observed. Studies in progress are being directed to a detailed investigation of the intermediates in testosterone production as an indication of possible metabolic blocks as well as kinetic studies related to production rates of metabolites (D. F. Johnson and N. Lamontagne).

Metabolism of C^{14}-Labelled Steroids by Adrenals from Rats with a Mammotropic Pituitary Tumor.

Adrenal metabolism of steroids, particularly progesterone, by adrenals subjected to long-term stress by ACTH, growth hormone and prolactin have been under study for some time. Earlier studies revealed both qualitative and quantitative differences in conversion of progesterone by the adrenals of MtT rats compared to normal rat adrenals. Studies in progress are designed to determine quantitative amounts of progesterone metabolites produced and kinetic relationships of biosynthesis of normal and stressed adrenals (D. F. Johnson and N. Lamontagne).

Steroid Transformations by Tetrahymena Pyriformis

Studies by others have demonstrated that a variety of steroids of mammalian origin, as well as steroid derivatives inhibit the growth of T. pyriformis, and that this growth inhibition is readily reversed by the addition of cholesterol to the medium. Of special importance in T. pyriformis meta-

bolism is the lipid tetrahymenol, a pentacyclic triterpenoid alcohol produced in the organism by a direct, non-oxidative, proton initiated cyclization of squalene. T. pyriformis grown without added steroids produces tetrahymenol as its major unsaponifiable product. Where steroids such as dehydroepiandrosterone are added to the culture an accumulation of squalene occurs with a corresponding decrease in growth rate of the organism. Since the focus of attention has always been on the action of steroids on tetrahymena, it was decided that the reverse study, i.e., the effect of tetrahymena on steroids would give additional information on the role of steroid metabolism in T. pyriformis. Radioactive steroid precursers have been added to T. pyriformis in the exponential phase of growth and the radioactive metabolites will be examined by chromatographic methods (N. Lamontagne and D. F. Johnson).

Chemical Structure - Biological Activity Correlations in the Enzymatic Reactions of Catechol-O-Methyltransferase (COMT) with Different Substrates

An attempt is being made to correlate chemical structure of COMT substrates with: a) the products of the enzyme substrate reaction (the meta/para ratio of the O-methyl ethers); b) Km and c) Vm. Statistical methods and computer techniques are being used in an attempt at graphical interactive molecular orbital minimal energy calculation via compilation of suitable computer programs, and regression analysis of the minimal energy conformations of substrates compared with biological parameters in the catechol-O-methyltransferase series. Similar correlation studies are underway in the N-substituted morphinan series (R. Katz).

Synthesis of Ecdysone-Like Compounds from Carpesterol

New compounds are desired having ecdysone-like moulting hormone activity, antiecdysone activity or chemosterilant activity on insects. Several such compounds were synthesized, starting with carpesterol, by a 6-step reaction series and include: 4α-methyl-(24R)-ethyl-coprost-6-on-7-ene-3β,14α,22R-triol, 4α-methyl-(24R)-ethyl-coprost-6,22-dion-7-ene-2β3β,14α-triol and 4α-methyl-(24R)-ethyl-coprost-6-on-7-ene-2β3β14α,22R-tetrol. Biological assay is in progress (G. Kusano and Y. Sato).

Photochemical Reactions of Certain Derivatives of Indole and Related Heterocyclic and Carbocyclic Compounds

Photolysis of N-chloroacetyltryptamine, N-chloroacetyl-3-(3'-aminopropyl)-indole, and N-3-(4'-aminobutyl)indole with mercury-vapor lamps results in the formation of high-melting dehydrohalogenation products in yields of 50%, 30%, and 20% respectively. The products are thought to be the tricyclic lactams resulting from cyclization in the 4-position of the indole moiety. Infrared spectral data, however, do not confirm the expected structural assignments. NMR studies are in progress with suitable derivatives to confirm final structural assignments to the dehydrohalogenation products. Additional investigations of photodehydrohalogenation are being conducted with N-chloroacetyl-2-(α-naphthyl)ethylamine as a prototype of polycyclic aromatic systems.

Kinetic data is obtained by an automatic phototitration technique, developed in the course of this work, and is providing useful information on the course and mechanisms of this reaction (C. M. Foltz).

Constituents of Solanum Xanthocarpum

Biologically active compounds affecting insect physiology are of considerable significance in the control of these insects and in the general field of insect biochemistry. A systematic chromatographic investigation of ligroin and alcoholic extracts from S. Xanthocarpum has resulted in 9 new steroidal compounds together with 11 known neutral steroids. The structures of the new compounds were determined through interconversion from carpesterol. Although solamargine, a steroidal alkaloid glycoside, has some larvaecidal activity, the mixture of solamargine and other glycosides is more active. A possible synergistic effect is suggested and under study (G. Kusano and R. Katz).

Alkaloids from Solanum Congestiflorum (Natri)

Identification of four alkaloid aglycons from S. Congestiflorum revealed an unusual unsaturated piperidino moiety on C-20, a possible biosynthetic intermediate of Solanum and Veratrum alkaloids. Studies of the glycosides have revealed some interesting variations in sugar moiety and sequence and are being investigated further (R. Katz, D. F. Johnson and Y. Sato).

Pyramidal Isomerism of Solasodine and its Derivatives

Solasodine shows some chemical properties different from its C_{22}, C_{25} isomer tomatidinol. This difference may result from the fact that solasodine has a specific pyramidal isomerism. This phenomenon may occur in other heterocyclic compounds, and general methods of differentiating these pyramidal isomers would be useful. Detailed NMR analysis is in progress to support the hypothesis of pyramidal isomerism in solasodine and its derivatives (G. Kusano, H. J. C. Yeh and Y. Sato).

SECTION ON PHARMACODYNAMICS

Studies on Pharmacodynamic Amines and Enzymes Involved in Their Metabolism

The 3-thio analog of dopamine was a potent irreversible inhibitor of catechol-O-methyltransferase in vitro but not in vivo. Inhibition was caused by the formation of a disulfide bridge to a sulfhydryl group in the active site of the enzyme and could readily be reversed with dithiothreitol or glutathione. In addition, the 3-thio analog of dopamine was i) a substrate of mitochondrial monoamine oxidase and a competitive inhibitor with tyramine as substrate, ii) a potent noncompetitive inhibitor of dopamine-α-hydroxylase, iii) a noncompetitive reversible inhibitor of phenethanolamine-N-methyltransferase. The compound did not cross the blood brain barrier, caused significant release of cardiac norepinephrine at high dosages and was rapidly eliminated in free form and as conjugates of itself and of the alcohol and acid formed by deamination (Lundstrom, Creveling).

Accumulations of cyclic AMP elicited in brain slices by tricyclic psychotropic drugs such as imipramine and chlorpromazine are mediated by a prior decrease in ATP levels and resultant "release" of adenosine. Tissue levels of ATP decrease presumably due to the interference with mitochrondrial oxidative phosphorylation caused by this class of drugs (Huang).

Rat glioma cells incorporate [^{14}C] adenine into nucleotides that subsequently can be converted to cyclic AMP in the presence of norepinephrine. The phosphodiesterase inhibitors, papaverine and isobutylxanthine, effectively enhance the stimulatory effect of norepinephrine. Elevated levels of cyclic AMP in these cultured cells elicits an apparent increase in phosphodiesterase activity. In contrast to results with brain slices, repetitive accumulations of cyclic AMP can be elicited during a series of restimulations of glial cells with norepinephrine. In addition, a large portion of the [^{14}C] adenine is incorporated in glioma cells into intracellular compounds that do not serve as precursors of [^{14}C] cyclic AMP (Schultz).

The order of relative activity of various local anesthetics as antagonists of the veratridine-elicited accumulation of cyclic AMP in brain slices was consonant with the order of their relative toxicity in clinical use for spinal anesthesia (Daly).

The Mechanism of Enzymatic Hydroxylation and the Role of Reactive Intermediates in Drug Metabolism

Cytochrome P-450 and P-448 fractions isolated from rat liver contain high levels of epoxide hydrase. A reconstituted P-450 system metabolizes naphthalene to equal amounts of naphthol and dihydrodiol via the intermediate naphthalene oxide. The monooxygenase and hydrase enzymes appear more closely associated in the P-448 system, where the predominant product from naphthalene is the dihydrodiol. Evidence for the presence of tightly-coupled membranal monooxygenase-hydrase systems that convert arenes to dihydrodiols has also been obtained in microsomal preparations from guinea pig livers (Oesch).

Levels of epoxide hydrase in inbred and hybrid C57 BL/6N and DBA/2N mice are similar and in contrast to monooxygenase activity are not inducible with 3-methylcholanthrene. Tumorigenesis was not related to induction of aryl monooxygenase activity in these mice (Oesch).

Epoxide hydrase activity towards styrene oxide, naphthalene oxide and benzene oxide is induced substantially in rats by pretreatment with phenobarbital and slightly by pretreatment with 3-methylcholanthrene. Attempts to inhibit epoxide hydrase activity in vivo with potent uncompetitive and noncompetitive epoxide inhibitors were unsuccessful. Such compounds do reduce glutathione levels in vivo, but this reduction had no significant effect on the arene oxide-mediated hepatotoxicity of chlorobenzene (Oesch).

The metabolism of various deuterated substrates has been studied with hepatic microsomes and *in vivo* in rats. Phenol formation occurs in most cases with the absence of a primary isotope effect. However, formation of the *meta*-hydroxylation product, 3-nitrophenol, from nitrobenzene exhibits an isotope effect k_H/k_D of 1.5, indicating that an arene oxide intermediate is not involved (Daly, Jerina).

An oxidative model system consisting of aqueous thiosalicyclic acid ferrous ions and oxygen has been studied further with naphthalene. In addition to naphthol, both *cis*- and *trans*-dihydrodiols and an unusual epoxide, 2,3-epoxy-4-hydroxy-1-tetralone, were isolated. Isotopic studies with O_{18} enriched water and oxygen gas provide evidence that both *cis*- and *trans*-diols are formed by the same reaction pathway, which does not involve an intermediate epoxide or dioxetane (Jeffrey, Jerina).

The mechanism of rearrangement and the inherent stability of arene oxides are crucial to an understanding of the toxicity, carcinogenicity and metabolism of such intermediates. A variety of deuterated and methylated arene oxides have been prepared and the kinetics of aromatization to phenols measured. The results provide the basis for predictions of arene oxide stability and have revealed a new mechanism for the NIH Shift which involves the intermediate formation from certain arene oxides of a 1,4-dihydrobenzene derivative. Synthetic approaches to arene oxides of polycyclic aromatic hydrocarbons are under exploration (Yagi, Jerina).

The course of addition of various nucleophiles to benzene oxide and naphthalene oxide has been elucidated, thus providing information on the potential reactions of such arene oxides with nucleophilic moieties of macromolecular tissue constituents (Jeffrey, Jerina).

Mono- and di-oxygenase activity have been further investigated in fungi and bacteria. Isotopic labeling with oxygen-18 established *cis*-dihydrodiol formation from naphthalene as typical of the action of a dioxygenase. The absolute stereochemistry of several such dihydrodiols is being investigated in terms of the fundamental basis for circular dichroism and optical rotatory dispersion. Bacterial and fungal systems exhibit monooxygenase activity as demonstrated by the NIH Shift during phenol formation, the isolation of *trans*-dihydrodiol from naphthalene and the incorporation of oxygen-18 into phenols from molecular oxygen. Fungi contain the only known monooxygenases that appear to effect the formation of oxirane-substituted arene oxides. The fungi, *Cunninghamella baineri*, has a nonspecific enzyme system remarkably simple to the P-450 drug metabolizing system of liver (Jeffrey, Jerina).

Novel findings regarding the fundamental nature of through space coupling observed in nuclear magnetic resonance spectroscopy have been made using ^{13}C and ^{15}N oxaziridines, imines and nitrones. In addition, the first known *cis*-aldimine has been prepared (Jerina).

Substantial improvement in the isolation and purification of catechol-O-methyltransferase was accomplished by use of affinose column chromatography. A preparation of enzyme with 80% purity was attained. This material was incubated with double-labeled radioactive S-adenosylmethionine in the absence of catecholic substrate. The enzyme on separation from unreacted S-adenosyl methionine by Sephadex chromatography was found to contain radioactive methyl groups which appeared to be partially transferred to catechols on subsequent incubation (Creveling).

The effects of all the trihydroxyphenethylamines and certain tetrahydroxyphenethylamines on the in vivo uptake and release of radioactive norepinephrine in mouse have been measured. 2,4,5-Trihydroxyphenethylamine (6-hydroxydopamine) and the 2,3,5-analog cause both initial release of norepinephrine and long term destruction of adrenergic binding sites. The 3,4,5 and 2,3,4 analogues are active only in terms of causing initial release, while the 2,4,6 and 2,3,6 isomers are relatively inactive in this system. The technique of labeling with [^{3}H]-norepinephrine has also been adopted for studies on uptake, storage and release in both arteries and veins (Lundstrom, Creveling).

5,6-Dihydroxytryptamine was prepared and found to cause selective depletion of serotonin in the central nervous system after intraventricular and intracerebral administration of the drug. Initially, 5,6-dihydroxytryptamine causes some release of norepinephrine, but its long term effects appear due to selective destruction of serotonergic systems similar to the selective destruction of adrenergic systems evoked by 6-hydroxydopamine (Daly).

The Role of Cyclic Adenosine Monophosphate in the Central Nervous System

Cyclic AMP accumulates in guinea pig cerebral cortical slices during incubations with histamine, norepinephrine, serotonin, adenosine, veratridine and combinations of these agents. The increases in endogenous levels of cyclic AMP and the increases in [^{14}C] cyclic AMP derived from intracellular nucleotides are quite similar. An accumulation of cyclic AMP in response to histamine or a histamine-norepinephrine combination can be elicited only one time, except in the presence of adenosine. The phosphodiesterase inhibitors, papaverine and isobutylmethylxanthine, are much more effective than theophylline in enhancing the amine-stimulated increase in cyclic AMP in brain slices. Comparison of "specific activity" of adenine nucleotides and cyclic AMP suggests that [^{14}C] adenine is incorporated into morphological compartments of adenine nucleotides which represent less than 30% of the total nucleotides of the slice (Schultz).

The results of studies on the effect of adenosine on accumulation of cyclic AMP in the presence of adenosine antagonists or of compounds which prevent the uptake of adenosine into brain slices suggest that the site of action of adenosine is extracellular (Schultz, Huang).

Serial No. NIAMD-LC-1
1. Chemistry
2. Biochemical Mechanisms
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Oxidation Mechanisms in Metabolic Processes

Previous Serial Number: SAME

Principal Investigator: Louis A. Cohen

Other Investigators: Y. Kikugawa and Herman Yeh

Cooperating Units: Jack S. Cohen, DCRT-PSL-5.11

Man Years

Total: 1.3
Professional: 1.3
Other: 0

Project Description:

A study of the mechanisms by which energy derived from respiration is ultimately converted into labile bond energy, as in ATP; a study of the mechanisms by which molecular oxygen is activated for direct incorporation into substrate.

Objectives: To demonstrate that when oxygen is bound to metal-chelate systems, it is transformed from the triplet (diradical) state to the singlet (ionic) state and that the direct incorporation of oxygen into substrate involves the electrophilic attack of activated singlet oxygen. To study possible mechanisms for the conservation of oxidative energy by means of chemical models and to apply such knowledge to elucidation of the enzymatic pathways. To devise chemical models for the Mitchell hypothesis--that ATP formation can be coupled to a pH gradient and the free energy available therefrom.

Methods Employed: Infrared and ultraviolet spectroscopy are used to follow rates of reaction of intermediates and to determine structural characteristics. The latter method is also used for quantitative assay of labile materials. Nuclear magnetic resonance spectroscopy is used for structural elucidation of closely related materials. Various chromatographic prodedures, particularly on thin layer plates, are used extensively.

Major Findings: A series of conformationally constrained imidazolecarboxylic acids has been prepared. While these compounds show little tendency

to cyclize spontaneously, their anomalous chemical behavior demonstrated that, in some of their reactions, cyclic acylimidazoles must be intermediates. Anomalous pK_a values also show the effect of bringing charged groups into extreme proximity and of the removal of solvent from the electrostatic gap. To date, such compounds represent the most effective models for the changes in ionic properties which are believed to occur when a substrate becomes bound to an enzyme surface.

Significance to Bio-medical Research and the Program of the Institute: The mechanism by which molecular oxygen is carried into the cell, bound to hemoglobin, has been well elucidated. Its subsequent fate remains obscure. One immediate goal of this project is to determine the mechanisms by which oxygen is activated to a state in which it can attack a variety of relatively inert substrates.

Similarly, the function of ubiquinone in oxidative phosphorylation has yet to be elucidated. It has been shown that oxidative energy is utilized in the conversion of ADP to ATP in mitochondria, ubiquinone being an essential cofactor.

The present findings permit a novel role to be assigned to ubiquinone, Vitamin K and other quinone cofactors.

Controversy regarding the Mitchell hypothesis for mitochondrial synthesis of ATP has continued unabated, since no experiment has been devised to test its validity. The model system under study will be used to attempt to settle the question of whether ATP can be synthesized simply by a rapid change in pH.

Proposed Course: Studies will be continued on bond conservation of oxidative processes and efforts will be made to bridge the gap between the chemical model and the mitochondrion. Studies will be continued on the effects of considerable reduction in the electrostatic gap.

Honors and Awards: None

Publications: None

Serial No. NIAMD-LC-2
1. Chemistry
2. Biochemical Mechanisms
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Chemical Modification and Cleavage of Proteins

Previous Serial Number: SAME

Principal Investigator: Louis A. Cohen

Other Investigators: None

Cooperating Units: Robert Jerussi, FDA
Sidney Shifrin, NCI-GL & C
Vincent Marchesi, NIAMD-LEP-4

Man Years

Total: 0.3
Professional: 0.3
Other: 0

Project Description:

A study of the chemical modification and fragmentation of polypeptides and proteins by use of selective chemical and physico-chemical reagents. The elucidation of sequence, tertiary structure and protein topology by such methods. Development of techniques for the selective radiolabelling of membrane proteins.

Objectives: To effect the selective modification and splitting of complex peptides by chemical means. To demonstrate the utility of the techniques in splitting long peptide chains into shorter fragments, facilitating sequence determination. To utilize such methods for selective modification of function groups on the surface of a globular protein and thus permit the probing of the surface structure. To modify proteins by selective oxidation or reduction techniques.

Methods Employed: Ultraviolet spectroscopy is used to follow the course and extent of cleavage reactions. Infrared spectroscopy is used to elucidate the structure of reaction products. Paper and column chromatography, ion exchange and high-voltage electrophoresis are used to separate and purify polypeptide fragments. An automatic amino acid analyzer is used to follow the extent of chemical reaction and the purification of peptide fragments. Gas

chromatography assists in quantitative end-group assay. Various techniques are used for assay of enzyme activity, including spectral and automatic pH devices. Electrochemical oxidation is effected by use of controlled voltage equipment coupled with automatic pH control.

Major Findings: A polymer containing N-iodosuccinimide units has been developed and applied to the selective iodination of highly exposed tyrosine residues in proteins. Initial studies have been performed with ribonuclease, asparaginase and erythrocyte membranes. A major goal of this work is to provide a method for the limited labelling of membrane protein with radioactive iodine.

Significance to Bio-medical Research and the Program of the Institute: Despite the remarkable achievements of the past several years in the X-ray crystallography of proteins, there exists no satisfactory method for determining three-dimensional structure in solution, under conditions of enzymatic activity. The use of a chemically reactive polymer or a platinum electrode, capable of selectively and rapidly modifying functional groups only on the surface, may provide a solution to the problem.

The ability to label membranes in a selective and specific manner with radioactive iodine will serve to facilitate studies on the sites and mechanisms of hormone binding to tissues.

Proposed Course: Information obtained by selective iodination, oxidation and reduction of proteins will be used to provide further information on conformation in solution.

Honors and Awards: None

Publications:

Takahashi, S. and Cohen, L. A.: Facilitation of sodium borohydride reduction of esters of phenols and of acidic alcohols. J. Org. Chem. 35: 1505-1508, 1970.

Serial No. NIAMD-LC-3
1. Chemistry
2. Biochemical
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Cleavage of Peptide Bonds by Intramolecular Participation and Mechanisms of Action of Hydrolytic Enzymes

Previous Serial Number: SAME

Principal Investigator: Louis A. Cohen

Other Investigators: None

Cooperating Units: None

Man Years

Total: 0
Professional: 0
Other: 0

This project has been temporarily discontinued.

Serial No. NIAMD-LC-4
1. Chemistry
2. Biochemical Mechanisms
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Fluoro Analogs of Enzyme Substrates

Previous Serial No.: SAME

Principal Investigator: Louis A. Cohen

Other Investigators: Kenneth L. Kirk and Wakatu Nagai

Cooperating Units: Claude Klee, NIAMD-LBP
Jack S. Cohen, CR-PSL
Dr. Michael Monahan, Salk Institute, La Jolla, California

Man Years

Total: 2.0
Professional: 2.0
Other: 0

Project Description:

Development of new methods for the synthesis of fluoro analogs of biochemically significant compounds and examination of their properties as enzyme inhibitors and as potential drugs.

Objectives: Fluoro analogs of steroids, purines, pyrimidines and amino acids have been synthesized and tested, several having been found valuable in arthritis and cancer chemotherapy. A larger number have not been prepared because of the limited routes available for the introduction of fluorine. Despite the ubiquitous role of imidazoles in biological systems (RNA, DNA, histidine, histamine, etc.), no fluorinated imidazole has yet been prepared. The initial purpose of this work was to develop a general method suitable for the preparation of fluoroimidazoles, to prepare a series of such compounds, and to test their abilities to serve as enzyme substrates or inhibitors and as replacements for histidine in proteins or for purines in DNA and RNA.

Methods Employed: Ultraviolet irradiation is used as the key step in the introduction of fluorine. Infrared, ultraviolet, mass, and nmr spectroscopy are used to follow the course of reactions and to elucidate product structure. Various chromatographic procedures are used for analysis and purification of materials.

Major Findings: D-amino acid oxidase and hog kidney acylase have been used to resolve 4-fluoro-DL-histidine into its optical enantiomers. Histidine decarboxylase has also been used to prepare 4-fluorohistamine and histidine deaminase to prepare 4-fluorourocanic acid.

Painstaking chromatographic purification of azo derivatives has now led to the preparation of 2-aminohistamine and 2-aminohistidine, as well as their 2-fluoro analogs. Studies are in progress on the fluorine nmr spectra of various enzyme-substrate complexes, using fluoroimidazoles as substrate analogs. Incorporation of fluorohistidine into polypeptide hormones and into enzyme fragments has also been initiated.

Significance to Bio-medical Research and the Program of the Institute: Past experience with fluoro analogs (fluorocitrate, fluorocortisone, and fluorouracil) warrants a search for other medicinally useful fluoroanalogs. Recent findings on the value of histidine in arthritis therapy, of imidazolecarboxamide in skin cancer therapy, and of histamine as a false neurotransmitter emphasize the magnitude of directions in which fluoroimidazoles may be examined clinically.

Proposed Course: Fouoroimidazoles already prepared will be submitted for pharmacological testing and will be examined as enzyme substrate analogs. Additional and more complex compounds will be prepared for the same purpose. The photochemical method of synthesis will be applied to other types of heterocyclic systems, in addition to imidazole. Fluorine nuclear magnetic resonance spectroscopy will be used to study enzyme-substrate complexes with a view to aiding in the elucidation of mechanism of enzymes such as histidine deaminase.

Honors and Awards: None

Publications:

Kirk, K. L. and Cohen, L. A.: Photochemical decomposition of diazonium fluoborates. Application to the synthesis of ring-fluorinated imidazoles. J. Amer. Chem. Soc., 93: 3060-3061, 1971.

Serial No. NIAMD-LC-5
1. Chemistry
2. Biochemical Mechanisms
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: General Principles of Enzyme Catalysis and Simulation

Previous Serial Number: SAME

Principal Investigator: Louis A. Cohen

Other Investigators: Paul S. Hillery

Cooperating Units: None

Man Years

Total: 1.4
Professional: 1.4
Other: 0

Project Description:

A study of the principles used by enzymes to accelerate chemical reactions. Model compounds are designed and synthesized which are capable of undergoing various reactions at rates comparable to those of enzymes, and under the same reaction conditions. Recognition of the principal devices used by enzymes will permit the design of more effective drugs and enzyme inhibitors or substrates.

Objectives: The rates of enzyme-catalyzed reactions exceed those of simple test-tube analogs by factors of 10^{10}-10^{18}. In order to account for such a phenomenal effect, model compounds which duplicate one or more of an enzyme's special powers are studied to learn more about the vastly complex protein catalyst. In the belief that the principal enzymatic device is stereopopulation control (near perfect orientation of functional groups), model compounds have been designed and synthesized in which rotation of covalent bonds has been severely restricted. By bringing two functional groups into very close proximity and orientation, rates of reaction comparable to those of enzymes can be achieved in the test tube.

Methods Employed: Spectroscopic methods are used to elucidate the structures of synthetic products and to follow rates of reaction. Various chromatographic procedures are employed for analysis and purification.

Major Findings: In extensions of our earlier studies, 3,3,4-trimethyl-

and 3,3,4,7-tetramethylphthalide were prepared. These compounds are completely resistant to ring opening to the hydroxyacids, although the carbonyl group undergoes facile ^{18}O exchange. These lactones are the first members of a new class of substances in which entropic factors completely overwhelm those of enthalpy. The rate of ring closure is so rapid that it becomes impossible of measurement.

These results demonstrate that the proper positioning of a substrate on an enzyme active site, as well as its very close approach to functional groups, may well be the most important factors in enzyme catalysis.

Honors and Awards: The new field of stereopopulation control was described in lectures at the University of Pennsylvania, Philadelphia, Pennsylvania, on February 16, 1971; at the State University of New York, Buffalo, New York, on November 11, 1971; at the University of Maryland, Baltimore, Maryland, on December 3, 1971; and at Pennsylvania State College on May 25, 1971. In addition, four seminars on the subject were presented within NIH.

Publications:

Hillery, P. S. and Cohen, L. A.: Stereopopulation control: The apparent stabilization of a lactone to ring opening. J. Chem. Soc.: 403, 1972.

Serial No. NIAMD-LC-6
1. Chemistry
2. Carbohydrates
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Higher-Carbon Sugars and Their Derivatives

Previous Serial Number: same

Principal Investigator: N. K. Richtmyer

Other Investigators: None

Man Years

Total: 1
Professional: 5 months
Other: None

Project Description:

Objective: To evolve generalizations relating the physical and chemical properties of the groups of substances named in the project title to their configurations and conformations.

Methods Employed and Major Findings: The general programs of research outlined in previous Individual Project Reports was pursued. D-*erythro*-D-*galacto*-Octitol, isolated earlier from the avocado, was oxidized with *Acetobacter suboxydans* to give L-*glycero*-D-*manno*-octulose as a syrup. When treated with methanol in the presence of a strongly acidic cation exchange resin the octulose gave a methyl octuloside and a second substance which is presumed to be 2,7-anhydro-L-*glycero*-β-D-*manno* octulopyranose.

Proposed Course of Project: The project terminated with the retirement of the investigator on November 30, 1971.

Honors and Awards: The November 1971, issue of the international journal *Carbohydrate Research* was dedicated to honoring Dr. Richtmyer.

Publications:

Richtmyer, N. K.: Crystalline D-*glycero*-L-*gluco*-octulose, crystalline methyl D-*glycero*-α-L-*gluco*-octulopyranoside, and some related compounds. *Carbohyd. Res.* 17: 401-410, 1971.

Richtmyer, N. K.: A crystalline, nonreducing anhydrooctulose, presumable 2,7-anhydro-L-*glycero*-β-D-*manno*-octulopyranose. *Carbohyd. Res.* In press.

Serial No. NIAMD-LC-7
1. Chemistry
2. Carbohydrates
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Immunochemistry and Reactions of Carbohydrates

Previous Serial Number: NIAMD-LC-7

Principal Investigator: C. P. J. Glaudemans

Other Investigators: None

Cooperating Units: NCI 5556

Man Years

Total: 1
Professional: 1
Other: None

Subtitle: Carbohydrate antigen-binding myeloma proteins in mice (with Dr. M. Potter).

Project Description:

Objectives: In the last few years a number of myeloma proteins have been found to precipitate with a variety of antigens such as nitrophenyl proteins, group specific polysaccharides such as pneumococcal C-polysaccharides, and a number of lipopolysaccharides associated with gram-negative organisms, to name but a few. Some of these antigens need not be part of the capular material of the microorganisms naturally present in mice developing the plasma cytomas. For instance, one IgA protein investigated here was precipitable by an arabogalactan extracted from the hardwood bedding of the mice cages of germ free mice, (SAPC 10), another of the IgA proteins, which also reacted with galactan, came from mice fed on wheat (TEPC 191B). A third IgA protein (TEPC 521) was present in the sera of mice also fed a wheat diet. This last serum reacted with wheat extract, but not with pure arabogalactan from larchwood, as did the other two sera. The object of this study is to utilize the fact that plasmacytomas put out homogeneous immunoglobulins, so that, when and if an antigen is found for them, they can be used to study the interaction of haptenic groups on the antigen with a pure antibody-like protein. In this case a particularly interesting aspect is that the two anti-

galactan IgA proteins are immunoglobulins reacting with a completely neutral polysaccharide antigen.

Methods Employed: All modern and classical techniques are called upon for kinetic and inhibition work, and in the elucidation of the structure of antigens and the synthesis of substrates. These involve hydrolysis studies, glc, mass spectrometry, n.m.r. and synthesis, affinity chromatography involving hapten-coupled sepharose columns.

Major Findings: Precipitin reactions. The three proteins were initially detected in the routine screening procedure as precipitins to a crude extract of the Old Guilford mouse food diet. This is the diet used in the laboratory. Precipitin reactions were obtained with serum from mice with several transplant generations as well as the serum from the mouse in which the plasma cell tumor was induced. Active antigenic components were found in wheat components of the Old Guilford and Wayne Blox diets. As wheat is known to contain a variety of pentosans and glucans a variety of polysaccharides of plant origin were screened as well. The S10 and T191B myeloma proteins (but not T521) precipitated with arabinogalactan and gum ghatti. The GF BALB/c mouse in which the SAPC-10 plasmacytoma was induced was fed a diet that did not contain wheat or an antigen similar to the one in wheat. Germfree mice are housed in jars that contain a hardwood bedding. In view of the finding that larchwood arabinogalactan contained an antigen with S10 and T191B precipitated, an extract was prepared from the hardwood bedding. Both S10 and T191B precipitated with this extract. Analysis of the hydrolysate of the extracted polysaccharide showed the presence of arabinose and galactose.

Specificities of the S10 and T191B myeloma proteins. The structure of larchwood arabinogalactan has been extensively studied. This polysaccharide has a backbone of 1 → 3 linked β-D-galactopyranose residues with side chains containing either L-arabinose or 1 → 6 linked β-D-galactopyranoses. Gum ghatti contains similar side chains although this is a more complex polysaccharide. Attempts were made to identify low molecular weight mono- and oligosaccharides that would inhibit the precipitation of gum ghatti or arabinogalactan with the myeloma proteins. Seven methyl pyranosides and furanosides were obtained or synthesized and tested for their inhibitory capacity at concentrations of 0.2 M. The following compounds were noninhibitory: methyl α-L and β-L arabinofuranosides and arabinopyranosides, methyl β-D-xylopyranoside, methyl α-D-galactopyranoside, and methyl β-D-galactofuranoside. Methyl β-D-galactopyranoside was weakly inhibitory at 0.143 M. Three oligosaccharides derived from gum ghatti proved to be potent inhibitors. 6-O-β-D-galactopyranosyl-D-galactose, the corresponding 1 → 6 β-D-galactotriose and tetraose. The most potent inhibitor on a molar basis was the galactotetraose. All of the inhibition reactions were determined with arabinogalactan as the

antigen. When gum ghatti was used as antigen, at the lowest possible concentration the inhibitors were much less effective. This may be due to the difference of the spacing of the side chains on the two antigenic backbone molecules.

Specificity of the T521 myeloma protein. The T521 protein did not precipitate with arabinogalactan or gum ghatti indicating this protein had a specificity different from S10 and T191B. The T521 protein reaction was unusual in another way, mainly that it developed only in the cold. We suspected the antigen identified by T521 in the cold might be a protein. The warm diet-extracted antigen was digested with trypsin or with pronase for 2 hr and it was found that pronase but not trypsin completely destroyed the antigenicity of the T521 antigen while not altering the S10 and T191B antigens.

Purification. The myeloma proteins are optimally purified from ascites or serum by affinity chromatography, i.e., immunoabsorption and elution with hapten. Two immunoabsorbents were prepared both involving p-amino-phenyl-β-D-thiogalactopyranoside. The first one involved coupling the glycoside to agarose derivatized with a side chain ending in a carboxyl group: Agarose $-NH\ (CH_2)_3\ NH(CH_2)_3\ NHCO(CH_2)_2\ COOH$ (by using a water soluble carbodiimide according to the procedure of Cuatrecacas). This absorbent could bind IgA monomer from myeloma serum containing IgA proteins precipitable by a $\beta 1 \rightarrow 6$ galactan. Binding was very tight however, the elution with hapten did not dissociate the immunoglobulin from the immunoabsorbent. Elution with sodium tetraborate buffer (pH 10) did yield IgA.

The second immunoabsorbent was better, in that the pure IgA could be eluted from the immunoabsorbent by the use of hapten solutions. It's preparation was as follows: p-aminophenyl-β-thiogalactoside was coupled to Bovine Serum Albumin (BSA). The BSA-glycoside (which precipitated the anti-galactoside IgA's very strongly) was then coupled to Sepharose activated with cyanogen bromide. The resulting Sepharose-BSA-thiogalactoside was a very effective immunoabsorbent. After passing reduced serum or ascites through the column, the bound IgA could be removed by elution with a buffer solution of p-nitrophenyl-β-D-galactoside. Pure IgA monomer was obtained and amino acid sequencing is in progress at this time. It is already clear that the amino terminal sequence of the TEPC 191 kappa chains is quite different from the general sequences of other kappa chains obtained from plasma tumors of inbred Balb/c mice.

Significance to Biomedical Research and the Program of the Institute. Work on the interaction between antigens and pure immunoglobulins will greatly enhance our knowledge of the immune process and the actual configuration of the binding sites on immunoglobulin.

Proposed Course of Project: There are other myeloma proteins which precipitate with hemophilus influenta type b, or lipopolysaccharides from Salmonella. This laboratory is involved in the preparation and synthesis of a number of inhibitors (haptenic groups) connected with these immunoglobulins. Then these haptenic groups will be coupled to solid supports to furnish immunoabsorbents so that the pure immunoglobulins can be isolated. Following that these globulins will be studied for their binding with haptens, and attempts will be made at crystallization to allow x-ray diffractions studies.

Honors and Awards: None

Publications:

Glaudemans, C. P. J. and Fletcher, H. G., Jr.: 2,3,4,6 Tetra-O-benzyl-α-D-glucopyranose. In Whistler, R. L. and BeMiller, J. N (Eds.): *Methods in Carbohydrate Chemistry*. New York, Academic Press, 1972, vol. 6, pp. 373.

Glaudemans, C. P. J. and Fletcher, H. G., Jr.: The Mechanism of Formation of Furanosylhalides. *J. Org. Chem.* 36: 3598, 1971.

Potter, M., Mushinski, E., and Glaudemans, C. P. J.: Antigen Binding IgA Myeloma Proteins in Mice: Specificities to Antigens Containing β1 → 6 linked D-galactoseside Chains and a Protein Antigen in Wheat. *J. Immunol.* 108: 295, 1972.

Potter, M. and Glaudemans, C. P. J.: Homogenous Immunoglobulins from Mice that Bind Carbohydrate Antigens. In Colowick, S. P. and Kaplan, N. O. (Eds.): *Methods in Enzymology*. New York, Academic Press, 1972, in press.

Serial No. NIAMD-LC-8
1. Chemistry
2. Carbohydrates
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on the Synthesis of Carbohydrate Derivatives for Biomedical Research

Previous Serial Number: Same

Principal Investigator: H. G. Fletcher, Jr.

Other Investigators: H. W. Diehl, A. Hasegawa, R. K. Ness, E. W. Tracy, E. Zissis.

Cooperating Units: Dr. N. Pravdić, Department of Organic Chemistry, "Ruder Bosković" Institute, Zagreb, Yugoslavia (under P.L. 480). Mr. K. W. Freer, Pfanstiehl Laboratories, Inc. Waukegan, Illinois

Man Years

Total: 7.3
Professional: 4.3
Others: 3

Part A. Aldonic Acids and 2-Acetamido-2-deoxyaldonic Acids

Project Description:

Objective: To develop new techniques for the isolation and characterization of aldonic acids; to extend knowledge of the chemistry of these substances and, in particular, to throw some light on those aldonic acids that are derived from aminosugars.

Major Findings: Investigation has shown that dicyclohexylammonium salts of aldonic acids may be prepared from aldono-1,5-lactones, aldono-1,4-lactones, metal aldonates and from the free aldonic acids. Although often accompanied by decomposition, the melting points of these substances are usually sharp and these salts appear to have some potential utility for the isolation and characterization of aldonic acids.

As mentioned in the report of 1970-1971, a crystalline dicyclohexylammonium 2-acetamido-2-deoxyaldonate can be prepared from 2-acetamido-2-deoxy-D-mannono-1,4-lactone as well as from the mixture of products which is obtained on the oxidation of 2-acetamido-2-deoxy-D-glucose with

unbuffered aqueous bromine. Earlier evidence, based wholly on various chromatographic and electrophoretic procedures, appeared to justify assignment of the D-gluco configuration to the dicyclohexylammonoum salt; through a series of reactions the salt has now been converted into 2-acetamido-2-deoxy-D-glucose and so its configuration may now be regarded as firmly established. An acidic product from the bromine-water oxidation of 2-acetamido-2-deoxy-D-galactose was isolated as a dicyclohexylammonium salt; conversion of this salt to the known 2-acetamido-2-deoxy-D-galactono-1,4-lactone demonstrated that salt formation in this case did not involve a configurational change. As part of this work, studies of the reduction of various 2-acetamido-2-deoxyhexonolactones were undertaken. Sodium amalgam was found to reduce a mixture of 2-acetamido-2-deoxy-D-gluconolactones to 2-acetamido-2-deoxy-D-glucose. Whether the aldose arose from the 1,4-lactone or from the 1,5-lactone (or from both) is not known. In any event, sodium amalgam failed to reduce either 2-acetamido-2-deoxy-D-mannono-1,4-lactone or 2-acetamido-2-deoxy-D-galactono-1,4-lactone. No rationalization of this unusual (if not unique) observation has been formulated. It may be noted in passing that 2-acetamido-2-deoxy-D-mannono-1,4-lactone may be reduced with sodium borohydride to 2-acetamido-2-deoxy-D-mannitol. Optical rotations in molybdate and acidified molybdate solution were shown to be useful for distinguishing this glycitol from its D-gluco epimer.

Significance to Biomedical Research and the Program of the Institute: Inasmuch as 2-acetamido-2-deoxy-D-glucono-1,5-lactone (and, perhaps, the corresponding 1,4-lactone) is an inhibitor of β-N-acetylglucosidase it is of interest to see whether this substance inhibits either of the two hexosaminidases involved in the human metabolism of sphingolipids. Dicyclohexylammonium 2-acetamido-2-deoxy-D-gluconate has been used as a source of the two 2-acetamido-2-deoxy-D-gluconolactones which were supplied to other NIH investigators for enzymological studies.

Part B. Unsaturated Aminosugars

Project Description:

Objective: To investigate the chemical properties of a new and unique class of carbohydrate derivative, the N-acyl and di-N-acylenamines, discovered earlier in this laboratory.

Major Findings: An earlier work, substances such as 3,4,6-tri-O-acetyl-2-(N-acetylacetamido)-1,2-dideoxy-D-arabinohex-1-enopyranose were shown to be susceptible to attack by nucleophiles in the presence of a trace of strong acid. Further study of this type of reaction has shown that such unsaturated aminosugar derivatives undergo stereospecific rearrangements when heated in the presence of an acid catalyst alone. This advance has made available a variety of 2-amino-2,3-dideoxyhex-2-enopyranose derivatives and these

have been used as substrates in a study of the behavior of unsaturated aminosugar derivatives in the presence of hydrogen and a palladium catalyst. While unsaturated acetamido sugars are readily reduced to acetamido sugars, the presence of a di-N-acetylamino group, attached to a carbon atom bearing a double bond, appears to inhibit totally the catalytic reduction of the double bond. Other changes can, however, take place, notably hydrogenelytic reactions and allylic shifts. These are under investigation.

Significance to Biomedical Research and to the Program of the Institute: The research project is designed to afford the type of basic information which will be required in a future endeavor aimed at the synthesis of novel materials of potential biochemical interest.

Part C. The Synthesis of Ribofuranosides and, especially of Ribonucleosides.

Project Description:

Objective: To devise a simpler and more practical procedure for the synthesis of β-D-ribofuranosides and, particularly, ribonucleosides.

Major Findings: Two procedures were devised for the synthesis of 2,3,5-tri-O-benzoyl-1-O-(p-nitrobenzoyl)-β-D-ribofuransoe and the utility of this substance for the synthesis of D-ribofuranosides was demonstrated.

Significance to Biomedical Research and to the Program of the Institute: The ribonucleosides and their relatives are such biochemical importance that my improvement in the preparation of these substances may be regarded as a useful contribution.

Honors and Awards: None

Publications:

Rabinsohn, Y. and Fletcher, H. G., Jr.: Synthesis of a Ketose from Partially Benzylated Aldose D-threo-pentulose from 2,3,5-tri-O-benzyl-D-arabinofuranose. In Whistler, R. L. and BeMiller, J. N. (Eds.): *Methods in Carbohydrate Chemistry*. New York, Academic Press, 1972, vol. 6, pp. 238-240.

Pravdić, N. and Fletcher, H. G., Jr: The Oxidation of 2-acetamido-2-deoxyaldoses with Aqueous Bromine. Two Diastereoisomeric 2-acetamido-2,3-dideoxyhex-2-enono-1,4-lactones from 2-acetamido-2-deoxy-D-glucose, -Mannose, and Galactose. *Carbohyd. Res.* 19: 339, 1971.

Pravdić, N. and Fletcher, H. G., Jr.: The Oxidation of Partially Substituted 2-acetamido-2-deoxyaldoses with Methyl Sulfoxide-acetic Anhydride. Some 2-acetamido-2-deoxyaldonic Acid Derivatives. *Carbohyd. Res.* 19: 353, 1971.

Ness, R. K., Fletcher, H. G., Jr., and Freer, K. W.: 2,3,5-Tri-O-benzoyl-1-O-(p-nitrobenzoyl)-ß-D-ribofuranose, a Convenient Reagent for the Preparation of 2,3,5-tri-O-benzoyl-D-ribofuranosyl Chloride and Bromide. *Carbohyd. Res.* 19: 423, 1971.

NIAMD-LC- 9
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: 1) The photolysis of N-chloroacetyl-N-methyl-1-3-(m-hydroxyphenyl)butylamine
2) Synthesis and pharmacological evaluations of 3,6-dimethyl-1,2,3,4,5,6-hexahydro-8-hydroxy-3-benzazocine and its 3-alkyl analogs
3) An alternative synthesis for 5-m-hydroxyphenyl-2-alkylmorphans
4) Synthesis and pharmacological evaluations of (+)-5-m-hydroxyphenyl-2-allylmorphan and its 2-cyclopropyl, 2-n-propyl analogs
5) Synthesis and biological studies of various 4-substituted catechols.

Previous Serial Number: Same

Principal Investigator: Helen H. Ong

Other Investigators: Everette L. May, Tokuro Oh-ishi, John W. Daly, and C. R. Creveling

Cooperating Units: A, LC, Section on Pharmacodynamics

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objectives: 1) To explore the applicability of the intramolecular photocyclizations developed by B. Witkop, et al., in the synthesis of medium sized (8-membered) benzazocines. 2) To evaluate pharmalogically the benzazocines as narcotic antagonists and/or agonists. 3) To develop a more practical synthesis for m-hydroxyphenyl-2-methylmorphan and its 2-alkyl analogs. 4) To study the mode of action of COMT with a variety of substrates.

Methods Employed: Synthetic techniques, chemical degradations, tlc, glc, mass, nmr, uv, and ir spectroscopy.

Major Findings: 1) m-Methoxyacetophenone reacted with formalin and N-methylbenzylamine to give β-(N-benzyl-N-methyl)-m-methoxypropiophenone in 90% yield. Treatment of the aminoketone with an excess of methyllithium gave the racemic ter. alcohol which was dehydrated with mesyl chloride and pyridine to give a mixture of cis and trans olefinic amines. Hydrogenation of the isomeric mixture with Pd black and acetic acid led to simultaneous saturation of the double bond and removal of the benzyl groups. Refluxing of the reduced product with conc. HBr gave 2-(m-hydroxyphenyl)-N-methyl-butylamine in 78% yield. The N-chloroacetyl derivative of this compound was prepared by reacting with chloroacetyl chloride in the presence of triethylamine (chloroacetic acid anhydride did not work in this case). Photolysis of the chloroacetamide was carried out with a Hg lamp, (Vycor filter) in aqueous methanol. para-Ring closure was observed to be the major reaction pathway, responsible for 30-35% of the products. When a corex filter was used, the principal product was N-acetyl-N-methyl-3-(m-hydroxyphenyl)butylamine, resulting from homalytic cleavage of the C-Cl bond and subsequent H abstraction.

2) Acetylation of the photocyclized compound, followed by reduction with LAH, gave 3,5-dimethyl-1,2,3,4,5,6-hexahydro-8-hydroxy-3-benzazocine in 78% yield.

3) A number of hitherto unknown 4-substituted catechols were prepared. These include 4-trifluoromethyl-, 4-fluoro-, 4-(N-benzamido)-, 4-(benzene sulfonamido)- and 4-carbomethoxymethyl catechols.

4) The sodio derivative of 2-(m-methoxyphenyl)cyclohexanone ($NaNH_2$ in refluxing benzene) reacted with ethyl bromoacetate at 0°C to give 2-(m-methoxyphenyl)-2-carbethoxymethyl cyclohexanone in 85% yield. The latter was nitrosated with amyl nitrite and sodium ethoxide to give 65% of the α-isonitroso compound. Attempts have been made to selectively reduce the isonitroso group while keeping the keto function intact.

Significance: 1) Photocyclization of chloroacetamides has proved to be a useful tool in synthesizing medium-sized heterocyclic rings which are ordinarily difficult to obtain; tertiary chloroacetamides cyclized almost as well as secondary.

2) The 3-benzazocines so prepared might emerge as a new class of potent analgesics since they are structurally close to the 6,7-benzomorphans, (the only missing component being the methano bridge C_5 and C_9).

3) The meta/para product ratios obtained with the various 4-substituted catechols might shed light on the steric as well as electronic requirements for COMT substrates.

Proposed course of project: 1) To prepare 3-allyl, 3-cyclopropyl and 3-n-propyl analogs of 3,6-dimethyl-1,2,3,4,5,6-hexahydro-8-hydroxy-3-benzazocine.

2) To seek better conditions for the selective reduction of 2-carbethoxymethyl-6-isonitroso 2-(m-methoxyphenyl)cyclohexanone and to effect the lactamization of the resultant amine ester. Wolff-Kishner reduction of the keto lactam, followed by teatment with diborane should lead to the desired m-methoxyphenylmorphan.

Publications:

Ong, H. H. and May, E. L.: Iminoethanophenanthridines by the Pictet-Spengler Reaction. J. Heterocyclic Chem. 8: 1007, 1971.

Ong, H. H. and May, E. L.: Photocyclizations. II. Synthesis of Iminoethanophenanthridine (Seven-membered Ring) Homologs. J. Org. Chem. 37: 712, 1972.

Creveling, C. R., Morris, N., Shimizu, H., Ong, H. H., and Daly, J.: Catechol-O-methyltransferase IV. Factors Affecting meta- and para-methylation of Substituted Catechols. J. Mol. Pharm., in press 1972.

NIAMD-LC- 10
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: 1) Derivatives of D-aspartic acid as potential antitumor agents.
2) Isolation and identification of medicinally active ingredients from bark obtained from the mountains of Peru

Previous Serial Number: NIAMD-LC-15

Principal Investigator: J. Harrison Ager

Other Investigators: Everette L. May

Cooperating Units: Pharmacology Unit of this Section

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objectives: 1) Make derivatives of D-aspartic acid to test as asparagine synthetase inhibitors and antitumor agents for comparison with the L-series previously prepared and found to have in vitro antitumor activity (see previous project reports).
2) To isolate and identify possible active ingredients from Peruvian bark obtained by J. W. Daly from South America and claimed by the natives to alleviate arthritic conditions.

Methods Employed: Standard methods of organic, physical organic and analytical chemistry.

Major Findings: Bis amides of (unnatural) D-aspartic acid were prepared in 50-70% yields with 2 equivalents of either phenethylamine or piperidine and diisopropylcarbodiimide. Hydrogenolysis (Pd-C) followed by reduction of the resultant amino bis amides with LAH, B_2H_6 or $B_2H_6NaAl(OCH_2CH_2OCH_3)_2$ (Red-Al) gave 3-amino-1,4-diaminobutanes. Similar reduction of the N-carbobenzyoxy bis amides (carbamates) gave 3-methylamino compounds in good yield.

Optical rotations of these compounds and the L-enantiomers previously prepared indicate no racemization during the carbodiimide-induced amide preparation stage or during hydride reductions. However, some racemization may have occurred during the hydrogenolysis (with Pd-$BaSO_4$ in MeOH) reactions. More work needs to be done to determine this.

Some amido hydrazides of D-aspartic acid were prepared from N-carbobenzoxy-D-aspartic acid and hydrogenolysis (Pd-C). No further work is planned here because of lack of activity except, in one instance, low activity against asparagine synthetase (in the L-series).

Shredded (Peruvian) bark (71 g) was extracted (twice) with 2:1 water—ethanol, then with 95% ethanol. After removal of solvent (H_2O pump, bath temp. 45-50°) 31 g of shiny brown residue (almost completely water soluble) was obtained. Elemental analysis showed 48.44% C, 4.34% H, and no N (mol. wt 207, mass spec.). Later the $^m/_e$ varied from 207 to 305 indicating change in composition of the residue on standing. So far, thin-layer and paper chromatography (using many different solvent systems) have failed to give appreciable concentration of any component. Crude samples showed inappreciable analgesic activity to 200 mg/kg (mice, hot-plate, oral and sc administration) and low activity in the Nilsen test. At 500 mg/kg a single dose reduced carrageenan-induced foot-pad edema in the mouse by 49%, a significant result.

Significance: This basic and applied research falls within the framework of this Institute and makes available new compounds of pharamacologic interest.

Proposed course of the project: Further attempts will be made to isolate and identify the components from the Peruvian bark extract and to examine them for biological activity as pure compounds. Additional modification of the D-aspartic acid molecule will not be continued unless significant biologic activity is seen in this area. The study of the Mannich and base-catalyzed alkylation reactions on 9-oxo-9,10-dihydroanthracene will continue. Molecular modifications of the potent 2'-hydroxy-2,9-dimethyl-5-propyl-6,7-benzomorphan will be made in hopes of developing agonists-antagonists as improved analgesics or as candidates for deterrence of heroin abuse.

Publication: None

NIAMD-LC-11
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: 1. Antimetabolites
2. Instrumentation

Previous Serial Number: Same

Principal Investigator: T. D. Perrine

Other Investigators: None

Cooperating Units: Ira B. Tice (NIH, R-BEI)

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:
Objectives: 1. To develop the synthesis of polymers carrying metabolites, primarily for anti-leukemia studies. 2. To develop new and improved methods of instrumentation.

Major Findings: 1. We still have no data from Cancer Chemotherapy on the activity of P-132S and P-1311-71B (PEI and His. on PEI, respectively, their 134422 and 134423). These were to be tested in a bloodstream leukemia.

Of the compounds we have submitted to Sloan-Kettering, polyethyleneimine itself and a graft of Serine + histidine on a terpolymer of (maleic acid-vinyl pyrrolidone-p-hydroxystyrene) have shown the greatest activity to date. The inhibition is in the order of 90%.

Of the anti-collagenase compounds submitted, polytyrosine was ineffective, but the reasons were obscure, and apparently its insolubility interfered with tests for anti-collagenase activity.

With regard to synthetic studies on the preparation of vinyl pyrrolidones with functional groups, all efforts we made with the p-benzyloxyphenyl derivatives were abortive, and I doubt that this is a good blocking group. Attempts to reduce p-nitrophenyl succinimide with THF-BH_3 were very complex, altho this seems to work well in the non-nitrated series. One of the more interesting approaches, which we did not investigate, was the reduction of imides to the half amid-half aldehydes. It appears as if this is a good reaction, and if the aldehyde can be converted to the schiffs base with dimethylaminoethyl amine, followed by reduction, this might be a worth while approach to the target monomers.

2) With regard to instrumentation, we have concentrated on working out the bugs on the viscometer. There is no doubt that the method is inherently very precise. We have experimented with a variety of viscometers and automatic fill mechanisms. The most practical finding is that the automatic fill mechanism need not be thermostatted, since temperature equilibration of the liquid is very rapid, and can be accomplished in a small stainless steel helix.

For practical application to biomedical problems, the need is for a totally sealed system of 1 ml capacity.

Proposed Course of Projects: Terminated

Publications: None

NIAMD-LC -12
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Titles: 1) Chemical Structure - biological activity correlations in the N-substituted morphinan series.
2) Chemical Structure - biological activity correlations in the catethol O-methyltransferase (COMT) substrate series.
3) Graphical interactive nuclear magnetic resonance analysis program.
4) Transformations in the morphine series - magnetic anisotropy of a spiro oxirane ring in a codeinone derivative.
5) Synthesis of agonist-antagonists in the benzomorphan series.

Previous Serial Number: Same

Principal Investigator: Arthur E. Jacobson

Other Investigators: S. R. Heller, H. J. C. Yeh, R. Katz, L. J. Sargent, E. L. May.

Cooperating Units: NIH (DCRT, Heuristics Lab.)(Heller)

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objectives: 1) To determine whether chemical structure and biological (analgesic) activity could be related in the N-substituted morphinan series of compounds.

2) To correlate chemical structure of COMT substrates with a) the products of the enzyme-substrate reaction (the meta/para ratio of the O-methyl ethers); b) Km, and c) Vm.

3) To compile computer programs for the rapid and precise determination of chemical shifts and coupling constants in the nuclear magnetic resonance spectra of complex drug molecules, by direct and immediate operator interaction with a graphics computer (PDP-10).

4) To determine the structures (by nmr analysis) of possible analgesic antagonists in the codeinone series; to determine the conformation of 6-methylisocodeine, 6-methylcodeine, and a spiro oxirane derivative of codeinone by the nuclear Overhauser effect; to determine the magnetic anisotropy of the ring bonds in the spiro oxirane.

5) To synthesize new agonist-antagonists.

Methods Employed: The usual organic, instrumental and computer techniques.

Major Findings: 1) Chemical structure is relatable to the analgesic activity of N-substituted morphinans, whether determined by the Eddy hot-plate test or by heat stimulation of the mouse-tail, the latter being an essentially time-independent biological test screen.

2) The meta/para ratio of O-methyl ether reaction products from the various COMT substrates can be related to the chemical structures of these substrates before interaction.

3) Direct and immediate operator interaction with a graphics computer can result in the rapid initial and iterative determination of chemical shifts and coupling constants of protons in complex molecules by the use of the GINA program.

4) The conformations, about C-6, of 6-methylisocodeine, 6-methylcodeine and the spiro oxirane derived from codeinone were determined, the analgesic activity of these new compounds was measured, and the effect of the spiro oxirane ring was calculated.

Significance: 1) The determination of the physical-chemical parameters necessary for analgesic activity can give additional insight into overall class generalizations, and perhaps a lead into future synthetic work.

2) The classification of the physical-chemical parameters which determine substrate O-methylation can give some insight into the catechol O-methyl-transferase system.

3) A major saving in time and cost was achieved by the use of GINA; what ordinarily would take weeks can now be achieved in hours.

4) The effect of a change in conformation about C-6 in the codeine series was observable by the analgetic activity of the various compounds.

Proposed Course of projects: An attempt at graphical interactive molecular orbital (CNDO-2 and INDO) minimal energy calculations via compilation of suitable computer programs.

2) Regression analysis of the minimal energy conformations of substrates compared with biological parameters in the catechol O-methyl-transferase series, and in the analgetic field.

3) Electron density and biological activity correlations of suitable series of drugs.

4) The observation of drug-enzyme interactions by Fourier transform nuclear magnetic resonance spectroscopy from the viewpoint of drug design.

5) An attempt to obtain direct qualitative and/or quantitative biological analyses of narcotic antagonists by formation of an in-house screening procedure.

6) Synthesis of additional analgesic agonist-antagonists.

Publications:

Eddy, N. B. and Jacobson, A. E.: Agonist-Antagonists. Current Medical Dialog, 38: 220, 1971.

Jacobson, A. E.: Structure-Activity Relations of Analgesic and Addictive Potentials. Proceedings of the 15th Annual Meeting of the Eastern Psychiatric Research Association, in press, 1972.

Perrine, T. D., Atwell, E. L., Tice, I. B., Jacobson, A. E., and May, E. L.: Analgesic Activity as Determined by the Nilsen Method. J. Pharm. Sci. 61: 86, 1972.

Jacobson, A. E.: Narcotic Analgesics and Antagonists, in Chemical and Biologic Aspects of Drug Dependence, Mulé and Brill, (Ed.): Chemical Rubber Co. Press Division, Cleveland, Ohio, in press, 1972.

Sargent, L. J. and Jacobson, A. E.: Transformations in the Morphine Series. V. Reaction of Codeinone with Dimethyloxosulfonium Methylide: Structure and Analgetic Activity of the Product and Its Reduced Form. J. Med. Chem., in press, 1972.

Jacobson, A. E., Yeh, H.J.C., and Sargent, L. J.: Nuclear Magnetic Resonance Spectra of Codeine and Isocodeine Derivatives. The magnetic Anisotropy of a Spiro Oxirane Ring. Org. Magn. Resonance, in press, 1972.

NIAMD-LC-13
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Development of Synthetic Procedures for Alkaloids

Previous Serial Number: Same

Principal Investigator: Edward M. Fry

Other Investigators: None

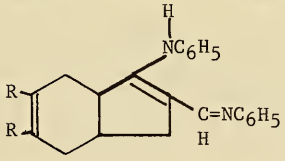
Man Years:
Total: 1
Professional: 1
Other: 0

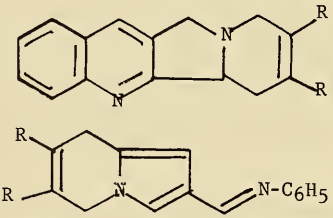
Project Description:

Objectives: The synthesis of nitrogen heterocycles.

Methods Employed: Standard methods of organic synthesis.

Major Findings: With a view to possible use in a camptothecin synthesis, a quaternary pyridinium salt gave the following compounds:

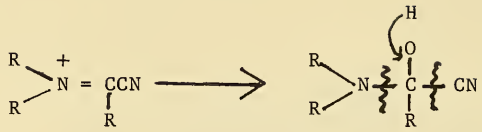




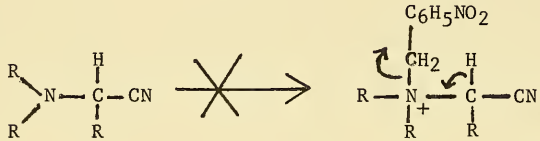
The ease with which nucleophildes add to the azomethine salt linkage suggested the use of amine oxides and dimethylsulfoxide in an attempted pyridone synthesis. This effort was unsuccessful.

A cyanczomethine salt should hydrolyze readily to give an amide and

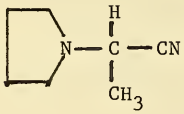
HCN or an amine and an α-keto nitrile,



but attempts to generate this system by N-quaternization followed by elimination failed.



The Polonovski approach gave a small yield of acetyl pyrrolidine with the model



Publications: None

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Cyclopropyldihydrocodeinone

Previous Serial Number: Same

Principal Investigator: Lewis J. Sargent

Other Investigators: Arthur E. Jacobson

Cooperating Units: Analytical Services Unit of this Section

Man Years:
Total: 1.0
Professional: 1.0
Others: 0

Project Description:

Objectives: The introduction of a cyclopropane ring at the 7,8-position of codeinone and the assessment of the derivative's analgesic powers relative to those of codeinone and dihydrocodeinone.

Methods Employed: Standard organic chemical procedures including mass spectrographic and NMR techniques.

Major Findings: The reaction of codeinone with an excess of dimethyloxosulfonium methylide in dry tetrahydrofuran led to a mixture which was separated (via column chromatography) into a small quantity (6% yield) of a novel codeinone derivative having the expected mass number, along with a quantity of codeine. NMR and IR analyses suggested the presence of an oxirane ring system in the new compound instead of the expected cyclopropyl ketone configuration.

Normally, α,β-unsaturated ketones (e.g., codeinone) react selectively with dimethyloxosulfonium methylide via methyl transfer to the α,β-unsaturated carbon-carbon double bond to form exo-cyclopropyl ketones. Moreover, it has been found that the methylide is also capable of reacting with the carbonyl function of saturated cyclic ketones to form oxiranes, and the observed anomalous reaction with codeinone to form an oxirane may be due to the steric constraints imposed by the rigid morphine ring system.

In addition to an exhaustive NMR study of the oxirane system, further evidence in support of this structure was obtained by reductive cleavage of the latter with lithium aluminum hydride to yield the previously unknown 6-methylisocodeine. Analgetic data for the two new substances were determined (in mice) and both were found to be somewhat more potent than codeine.

Significance: The preparation of two novel codeinone derivatives is further evidence of the protean character of the morphine ring system and adds measureably to the body of knowledge concerned with the structure activity relationships in this area.

Proposed course of project: Project completed.

Publication:

Sargent, L. J. and Jacobson, A. E.: Reaction of Codeinone with Dimethyloxosulfonium Methylide: Structure and Analgetic Activity of the Product and its Reduced Form. J. Med. Chem. in press 1972.

Jacobson, A. E., Yeh, H.J.C., and Sargent, L. J.: Nuclear Magnetic Resonance Spectra of Codeine and Isocodeine derivatives. The Magnetic Anisotropy of a Spiro Oxirane Ring. Org. Magn. Resonance, in press 1972.

NIAMD-LC- 15
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Respiratory Enzyme Inhibitors

Previous Serial Number: NIAMD-LC-18

Principal Investigator: James G. Murphy

Other Investigators: None

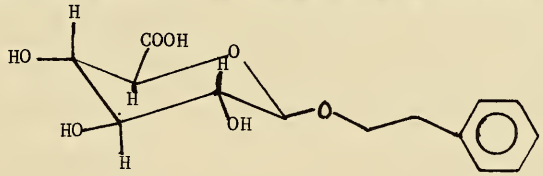
Cooperating Units: None

Man Years:
Total: .5
Professional: .5
Other: 0

Project Description:

Objectives: To seek reversible inhibitors for respiratory enzymes for possible therapeutic application.

Methods Employed and Major Findings: The direct method of glucuronidation was improved and applied to methyl-, phenyl-, and phenethyl-glucuronides. In the latter case, a pure crystalline potassium salt of phenethyl-B-glucopyranosiduronic acid (I) was characterized and isolated in 52% yield.



I

Using specific ion electrode technology, this glucuronide was shown to complex specifically with copper but not with Na^{+}, K^{+}, Ca^{+2}, Mg^{+2}, Fe^{+2}, Co^{+2}, Mn^{+2}, or Zn^{+2} at pH 7.8 in dilute aqueous solution.

Significance: Because of its special properties, I is being evaluated

for antiinflammatory properties (by Dr. May). Because Wilsons' disease is a disorder of copper metabolism, these results have been communicated to Dr. Jery D. Gardner (A:D&HD). Because malanoma tumors involve copper-dependent enzymes, samples of I have been forwarded to Dr. Harry B. Wood, (C:DD).

Proposed course of project: Terminated

Publications: Murphy, J. G.: Glucuronide Synthesis, Specific Complexing of Copper. J. Pharm. Sci., in press 1972.

NIAMD-LC-16
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Testing for Analgesic Activity and Dependence Liability

Previous Serial Number: Same

Principal Investigator: Everette L. May

Other Investigators: E. L. Atwell, E. McNeal, and N. B. Eddy (consultant)

Cooperating Units: Animal Production Section, NIH; Department of Pharmacology, University of Michigan

Man Years:
Total 1.5
Professional: 0.5
Other: 1.0

Project Description:

Objectives: 1) To determine analgesic activity, acute toxicity, narcotic antagonist activity and abuse potential of new compounds prepared in the Section on Medicinal Chemistry and in laboratories throughout the world. 2) To serve in an advisory capacity to the BNDD, FDA, WHO, SAODAP and the pharmaceutical industry.

Methods Employed: Hot plate and Nilsen methods of assay for analgesia and 24-hour acute toxicity determinations using in all instances male, white, Caeserian-Derived, General-Purpose mice. Abuse potential and properties of narcotic antagonism were determined in Rhesus monkeys and, if warranted, in man at the Addiction Research Center, Lexington, Kentucky.

Major Findings: A total of 75 compounds were submitted and assessed for analgesic activity, all by the hot-plate method, 46 by the Nilsen technique. These tests involved 166 dose-range finding experiments. Sixty-four were subjected to complete assay in the hot-plate, 30 by Nilsen. Acute toxicities were determined for eight. Of the 75 compounds (of widely varied structure) submitted, 25 were from the Section on Medicinal Chemistry, the remainder from the pharmaceutical industry. Several promising analgesics, potentially pure antagonists and interesting mixed agonists-antagonists have emerged from the past year's study.

Significance: Constitutes a concerted effort in attempts at the solution of the problem of drug addiction.

Proposed Course of Project: To continue essentially as is with possible addition of simple primary tests for physical dependence capacity, antagonistic action and antiinflammatory activity (all in the mouse).

Publications: Ong, H. H. and May, E. L.: Iminoethanophenanthridines by the Pictet-Spengler Reaction. J. Heterocyclic Chem. 8: 1007, 1971.

Ong, H. H. and May, E. L.: Photocyclizations. II. Synthesis of Iminoethanophenanthridine (Seven-Membered-Ring) Homologs. J. Org. Chem. 37: 712, 1972.

Thyagarajan, G. and May, E. L.: Improved Synthesis of 2-Benzyl-1,2,5,6-Tetrahydropyridines, Precursors of 6,7-Benzomorphans. J. Heterocyclic Chem. 8: 465 (1971).

Perrine, T. D., Atwell, E. L., Tice, I. B., Jacobson, A. E., and May, E. L.: Analgesic Activity as Determined by the Nilsen Method. J. Pharm. Sci. 61: 86, 1972.

May, E. L: Agonist and Antagonist Actions of Narcotic Analgesic Drugs. Synthetic Compounds. Symposium on Antagonists, July 12, 1971, McMillan, in press.

Eddy, N. B. and Jacobson, A. E.: Agonists-Antagonists, Current Clinical Dialog. 38: 330, 1971.

Eddy, N. B.: Agonists-Antagonists. Historical Overview. Symposium on Antagonists. July 12, 1971 McMillan, in press.

NIAMD-LC-17
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: 1) Synthesis of N-Derivatives of Ketobemidone
2) Synthesis of N-Derivatives of (±)- and (+)-phenylmorphan
3) Synthesis of 9-Methyl-6,7-benzomorphans and their derivatives.

Previous Serial Number: Same

Principal Investigator: Tokuro Oh-ishi (V.S.) and E. L. May

Other Investigators: H. Ong (project #2)

Cooperating Units: Pharmacology Unit and Section on Microanalytical Services and Instrumentation of this Laboratory

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objectives: 1) To synthesize N-derivatives (ethyl, propyl, butyl, amyl, hexyl, heptyl, allyl and cyclopropylmethyl) of ketobemidone and evaluate their analgesic and/or antagonistic activity. 2) To synthesize N-derivatives (propyl, allyl and cyclopropylmethyl) of (±)- and (+)-5-m-hydroxyphenylmorphan and evaluate their analgesic and/or antagonistic activity. 3) To synthesize 9-methyl-6,7-benzomorphans and derivatives by known or new routes and evaluate their analgesic activity.

Methods Employed: Reaction methods and techniques generally employed in organic chemistry.

Major Findings: 1) (a) 1-Methyl-4-(m-methoxyphenyl)-4-proprionyl-piperidine was synthesized from the corresponding nitrile according to the literature. Yield was improved up to double in the shorter reaction time. N-demethylation of the product was easily attained by reaction with ethyl chloroformate followed by hydrolysis in refluxing 20% aq HCl.
(b) Synthesis of the N-substituted derivatives described under objectives was accomplished in good yield. Tests for analgesic activity gave ED_{50}'s of 23.0, 4.5, 1.7, 0.31, 3.0 and 3.7 mg/kg respectively for N-ethyl through

N-heptyl, respectively. The N-amyl compound was three times as potent as ketobemidone and morphine; N-allyl was 1/3 as potent as morphine while N-cyclopropylmethyl (and the corresponding alcohol) are almost inactive by the hot-plate and Nilsen methods.

2) (a) The starting compounds, 5-m-hydroxyphenylmorphans, were synthesized from the corresponding 2,5-dimethyl compound by von Braun reaction followed by treatment with 48% aq. HBr. (b) Conversion of this NH compound, (+) and (±) to N-allyl, propyl and cyclopropylmethyl gave compounds of pethidine-like to codeine like or less analgesic activity as determined in both the hot-plate and Nilsen tests.

Significance: A general method to synthesize N-derivatives of phenylmorphans, norketobemidone was established. These compounds are candidates as agonists, antagonists or mixed agonists-antagonists for use in the fight against drug abuse.

Proposed course of the project: To continue 1 and 2, to complete evaluation of all compounds above for analgesic activity and antagonistic potency.

Publications: None

Serial No. NIAMD-LC-18
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Photochemical Oxidation and Reduction of Diterpenes

Previous Serial Number: NIAMD-LC-28

Principal Investigator: J. A. Waters

Other Investigators: B. Witkop

Cooperating Units: I. Karle, U.S. Naval Research Laboratory, Washington, D.C.

Man Years:

Total: 0
Professional: 0
Others: 0

This project has been terminated.

Publications:

Kondo, Y., Fourrey, J.-L., and Witkop, B.: Alkali sensitivity of 3-methylpyrimidine nucleosides. J. Am. Chem. Soc. 93: 3527-3529, 1971.

Fujimoto, Y., Irreverre, F., Karle, J. M., Karle, I. L., and Witkop, B.: Synthesis and X-ray analysis of cis-3,4-methylene-L-proline, the new natural amino acid from horse chestnuts, and of its trans isomer. J. Am. Chem. Soc. 93: 3471-3477, 1971.

Serial No. NIAMD-LC-19
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Anodic Decarboxylation of Glycidic Acids

Previous Serial Number: NIAMD-LC-29

Principal Investigator: J. A. Waters

Other Investigators: B. Witkop

Cooperating Units: None

Man Years:

Total: 0
Professional: 0
Others: 0

This project has been terminated.

Publications:

Waters, J. A. and Witkop, B.: Anodic decarboxylation of glycidic acids. J. Org. Chem. 36: 3232-3235, 1971.

Serial No. NIAMD-LC-20
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Selective Modification of Free and Bound Tryptophan

Previous Serial Number: SAME

Principal Investigator: Thomas F. Spande

Other Investigators: H. C. J. Ottenheym and B. Witkop

Cooperating Units: George Glenner, NIAMD-LEP

Man Years:

Total: 1.8
Professional: 1.3
Others: .5

Project Description:

Objectives: To determine the structure(s) of the reaction products from tryptophan and simpler indole models with the widely used histochemical reagent, Fast Red-B (1).

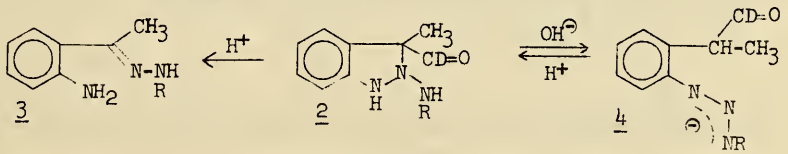
Methods Employed: NMR, UV and IR spectroscopy, mass spectrometry and thin layer and column chromatography.

Major Findings:

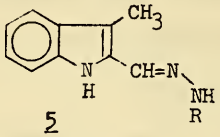
a) Simple indoles

The reaction with skatole was found to be pH-dependent. At pH 3.0, a 2-azo product predominates accompanied by minor amounts of the pyrrole-ring cleaved aldehyde (2), while at pH 7.0 the proportions of these two products were reversed. When 2-deuteroskatole was coupled at pH 3.0, the 2-azo product had lost deuterium while the aldehyde retained the label. 2 undergoes a facile Japp-Klingemann deformylation with dilute HCl to the hydrazone 3 and is reversibly converted to the deeply colored triazene anion 4 on treatment with alkali.

Scheme 1 (R = o-methoxy-p-nitrophenyl)



2,3-Dimethylindole at either pH 3.0 or 7.0 produces the hydrazone 5 as the sole product. This interesting and potentially useful reaction (hydrolysis affords 2-formylskatole, a formal oxidation) seems a new example of a small class of 1 → 3 rearrangements in the indole series.



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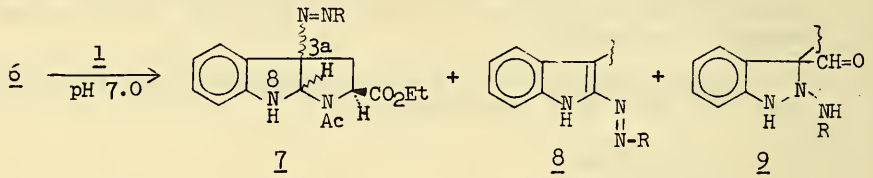
1,2-Dimethylindole with 1 produces a 3-azo derivative exhibiting no bathochromic shift with alkali. This confirms speculation in the literature that such shifts observed with 3-azo products from indole or 2-methylindole arise from removal of the indole N-H proton.

b) N-Acetyltryptophan ethyl ester (6)

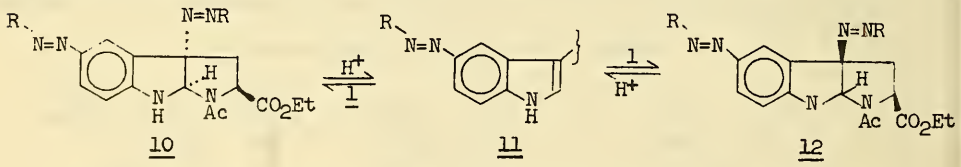
At pH 7.0, 6 affords as the major (60%) product, an unstable, unconjugated 1:1 azo compound 7 and minor amounts of the 2-azo derivative 8 and cleaved product 9. 7 is converted solely to 8 on acid treatment but disproportionates to 6 and a 2:1 azo compound 10 in methylene chloride or chloroform. A stable 8-acetyl derivative of 7 could be prepared. A UV



7 8 9

spectrum of 7 in weakly acidified ethanol is consistent with a mixture of 6 and $R\text{-}\overset{+}{N}\text{=}N$ and demonstrates the reversibility of the conversion 6 → 7.

At pH 3.0, 6 gives two 2:1 azo compounds (one identical to 10 above) and two 1:1 azo compounds (one identical to 8 above). The new 1:1 compound was shown to be the unusual 5-azo derivative 11, while the new 2:1 product (12) the 3a-epimer of 10.



10 11 12

11 results when either of the bis azo adducts 10 or 12 is treated with

acid in the presence of β-naphthol. Also when 11 is treated with 1 at pH 3 a mixture of 10 and 12 results. When 7 is treated with excess 1 at pH 3 (but not pH 7.0), 10 results.

The structure proof for 11 rests in its reduction ($S_2O_4^{--}$) and acetylation to 5-acetamido N-acetyltryptophan ethyl ester and comparison of its UV and NMR spectra with the model, 5-acetamidoindole.

Interestingly, no stable bis-substituted azo adducts from 6 (e.g. a 2,5-bisazo tryptophan derivative) could be detected, either in reaction of 1 with 11 or in the pH 3.0 reaction with 6. Presumably the inductive effect of the 5-azo substituent is responsible. The formation of 11 is, to our knowledge, the first example of an electrophilic substitution reaction in the indole series where an indole results which is substituted in the benzene ring but still retains an unsubstituted 2-position.

Significance to Biomedical Research and the Program of the Institute: We initially undertook the study of the reaction of diazonium salts with tryptophan and simpler indoles merely to demonstrate their reactivity and to caution workers in the affinity labelling of proteins that the widely used technique of coupling with diazonium salts very likely gives reaction at tryptophan as well as the expected reactions at histidine or tyrosine residues.

The unraveling of the structures of the products resulting from a tryptophan model extends the small number of examples of reactions whereby tryptophan undergoes an intramolecular cyclization - in this case in a very mild electrophylic substitution reaction of multiple steps, one of which is reversible. The production of 5-substituted tryptophan products is particularly exciting and makes possible (reduction, Sandmeyer type reactions) the direct preparation from L-tryptophan of derivatives (NH_2, OH, F, etc.) substituted in the biologically important 5-position.

The reaction of 3-substituted indoles incapable of such cyclizations still has possible biomedical utility. These should give rise at pH 7.0 to pyrrole ring-cleaved products analogous to 2 from which the original C-2 carbon atom can be easily extruded by acid treatment. This procedure could be of potential usefulness in biosynthetic studies.

Proposed Course: To study the reaction of N-acetyltryptamine in hopes of obtaining 5-azo substituted tryptamines. These should be convenient intermediates in new syntheses of serotonin, bufotenine and melatonin. To study the effect of methylation of the indole nitrogen on the course of the reaction. To investigate (with the collaboration of Dr. K. Kirk of this Institute) the practicability of converting 11 to 5-fluoro-N-acetyl-L-tryptophan ethyl ester (a derivative of the antimetabolite 5-fluorotryptophan) using a photolytic fluorination procedure.

Honors and Awards: None

Publication: Ottenheym, H. C. J., Spande, T. F., and Witkop, B.: The synthesis and reactions of a tetrachlorodioxopiperazine. J. Org. Chem. In press.

Serial No. NIAMD-LC-21
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Biosynthesis of Collagen: Inhibitors of Proline Hydroxylase; New Synthetic and Natural Analogs of Hydroxyproline

Previous Serial Number: SAME

Principal Investigator: B. Witkop

Other Investigators: None

Cooperating Units: S. Udenfriend, Roche Institute of Molecular Biology, Nutley, N. J.
A. Verbiscar, Institute of Drug Design, Inc., Sierra Madre, California
Isabella Karle, U. S. Naval Research Laboratory, Washington, D. C.

Man Years:

Total: 0
Professional: 0
Others: 0

This project has been terminated.

Serial No. NIAMD-LC-22
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Synthesis and Biochemistry of Potential Antiviral and Carcinostatic Nucleotides and Nucleosides

Previous Serial Number: SAME

Principal Investigators: P. Torrence and J. A. Waters

Other Investigators: B. Witkop, T. Nagamachi, Sam Baron, NIAID-LBV, Maxine Singer, NIAMD-LBM, C. Buckler, NIAID-LBV

Cooperating Units: Professor Lawrence Grossman, Brandeis University; Florence White, NCI-DE; Gerald A. LePage, Stanford Research Institute, Palo Alto

Man Years:

Total: 4
Professional: 3
Others: 1

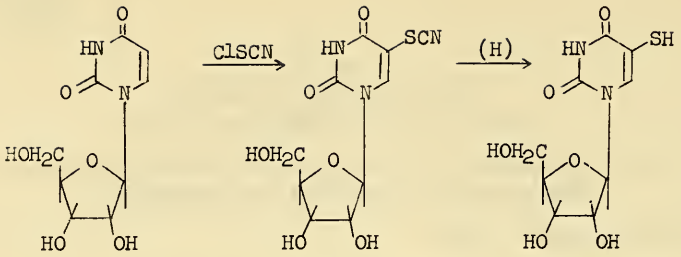
Project Description

Objectives: The aims of this project are to investigate the chemistry and biochemistry of both normal and "fraudulent" nucleosides with the objective of developing basic procedures and uncovering useful information which will lead to effective anti-viral and anti-cancer agents at the monomer (nucleoside) and polymer (polynucleotide) levels.

Methods Employed: Thin-layer, paper, ion-exchange and column chromatography, liquid scintillation, ultraviolet, visible, infrared, nuclear magnetic resonance, optical rotatory dispersion and mass spectroscopy.

Major Findings: The above objectives are presently being pursued at increasing levels of biochemical and chemical complexity.

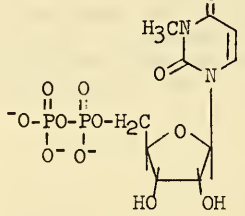
1. At the nucleoside level, additional "fraudulent" nucleosides are being developed which may be of use in themselves as anti-viral or carcinostatic agents and also, when polymerized, to give polynucleotides which may yield information regarding the physical and biological properties of nucleic acids. Of substantial potential is the following reaction sequence:



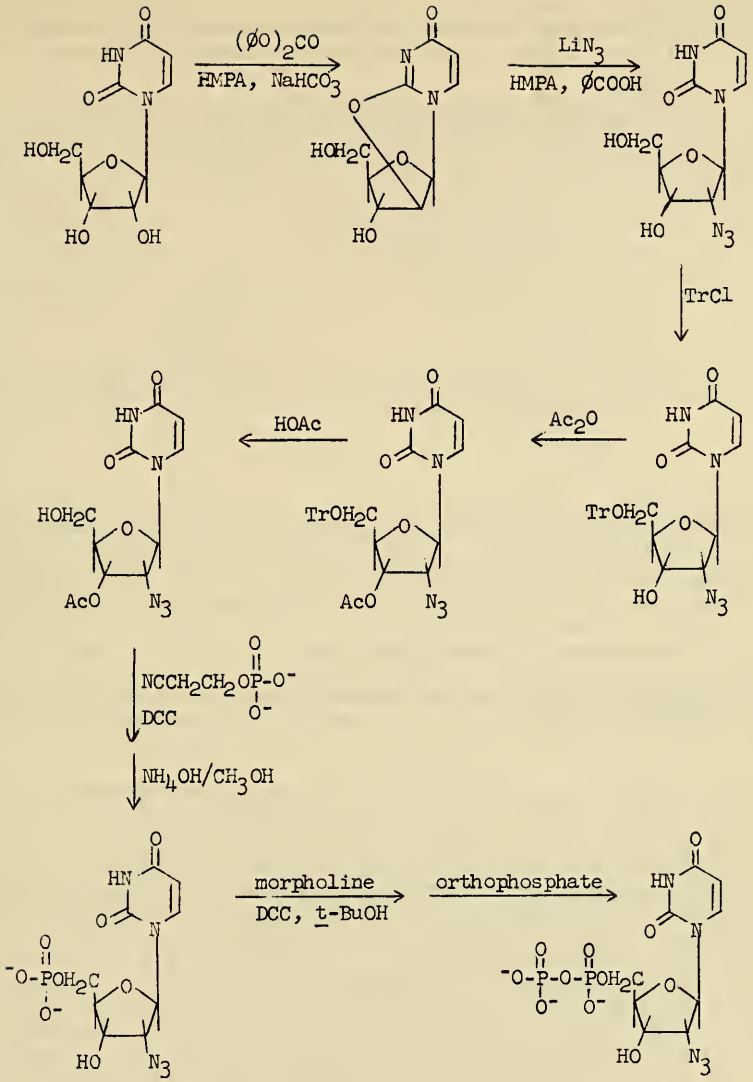
I II III

We have found that the conversion of I to II by chlorothiocyanogen (a pseudohalogen) proceeds in reasonable yield (20-30%). Compound II has been identified on the basis of elemental analysis as well as spectral (UV, IR, NMR) data. This conversion represents the first example of an addition-elimination reaction of a pseudohalogen with a pyrimidine nucleoside. Compound II will be screened for anti-viral and cytotoxic properties and, hopefully, polymerized to give poly 5-thiocyanatouridylic acid, which is of interest with respect to other aspects of this project (vide infra). Although no chemical studies have yet been performed, there is every reason to believe that II can be reduced (e.g., with mercaptoethanol and sodium dithionite) to III (5-mercaptouridine). III has previously been synthesized by a modification of the Hilbert-Johnson reaction and has shown inhibitory activity on preliminary testing in bacterial and tissue culture assays (J. Med. Chem., 13, 708 (1970)). In addition, 5-mercapto-2'-deoxyuridine is an effective antitumor agent (Molecular Pharmacology, 6, 621 (1970)). Thus, the above series of reactions would lead to a quick and convenient method for the synthesis of III, which could easily be applied to other precursors to give the deoxy, arabinosyl, lyxo and xylo derivatives (of both the 5-SCN and 5-SH) which may prove to be biologically active.

2. At the nucleotide level, this project involves the synthesis of mono- and diphosphates of nucleosides as intermediates in the polynucleotide phosphorylase catalyzed polymerization to give unusual polynucleotides. In connection with the sections listed below, we have synthesized 3-methyluridine diphosphate and 2'-azido-2'-deoxyuridine 5'-diphosphate by the following series of reactions. 2',3'-Isopropylidene-3-methyluridine was phosphorylated with pyrophosphoryl chloride and the resulting monophosphate was converted to the 5'-diphosphate through the morpholidate. 3-MethylUDP has been synthesized



3-MethylUDP

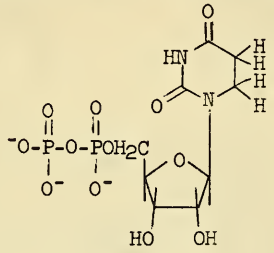


2'-Azido-2'-deoxyuridine
5'-Diphosphate

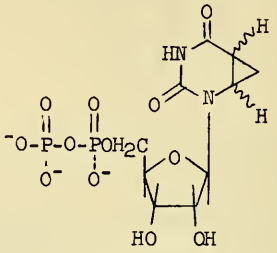
previously by another route. Moffatt (J. Org. Chem., 36, 250 (1971)) had

previously reported the synthesis of the free nucleoside, 2'-azido-2'-deoxyuridine. The above sequence involves the first reported synthesis of the 5'-mono- or 5'-diphosphate.

3. At the level of enzymes involved in polynucleotide synthesis, we have recently completed studies on the behavior of compounds A and B as substrates



A



B

for polynucleotide phosphorylase (see previous project report). Studies with these substrates have revealed useful information regarding the substrate specificity of polynucleotide phosphorylase as well as methods for the synthesis of nucleic acid copolymers containing "fraudulent" nucleosides. Several of the products arising from this study have been used in connection with studies on interferon induction (vide infra).

4. Polynucleotides. We have recently prepared a highly unusual polynucleotide which promises to play an important role in elucidation of the conformations of single- and double-stranded polynucleotides.

Poly 2'-azido-2'-deoxyuridylic acid (poly U_Z) was prepared by the polymerization of the corresponding nucleoside diphosphates (see section 2) with the enzyme polynucleotide phosphorylase and Mg^{+2} as cofactor. Mn^{+2} ion raised the yield of polymer by 10-20% but was not necessary for de novo synthesis. This behavior contrasts sharply with that of other nucleoside diphosphates modified in the 2'-position since such substrates usually require Mn^{+2} for polymerization. Poly U_Z prepared in the above manner had $s_{20,w} = 8.0S$ and gave only U_Z on enzymatic hydrolysis. As required by the absence of a 2'-OH function, poly U_Z was completely resistant to degradation by pancreatic ribonuclease. The thermal stability of poly U_Z is novel because with $T_m = 12°$ (both by UV and ORD) it surpasses $T_m = 6°$ for poly U under comparable conditions. Poly U_Z forms a 1:1 complex with poly A as evidenced by the eutectic at 50 mole %. This complex underwent a smooth cooperative transition with $T_m = 59°$, not markedly different from poly A·poly U.

Poly U_Z is the first and only example of a stable secondary structure in a single stranded polynucleotide without a 2'-oxygen function. Its physical properties conclusively demonstrate that neither a 2'-OH nor a 2'-O function

is necessary for secondary structure in polyribonucleotides vis-a-vis polydeoxyribonucleotides. More detailed physical studies on poly U_Z should shed light on the role of the 2'-OH in RNA.

5. The Induction of Interferon by Synthetic Polynucleotides. Interferon, discovered in 1957 by Issacs and Lindenmann, appears to be a significant natural defense system against viral diseases in man and animals. Because of the obvious clinical value of interferon, the difficulties involved in obtaining large amounts of human interferon, and interest in the mechanism of interferon induction, substantial attention has been focused on synthetic interferon inducers, such as the polynucleotide duplex, poly I·poly C. Aside from, or possibly connected with the antiviral activity of such synthetic polynucleotides, is the fact that such materials can inhibit growth of a large variety of animal tumors.

The following hypotheses, with varying amounts of supporting evidence, have been offered regarding the requirements which are involved in determining the efficacy of a polynucleotide as an interferon inducer:

1. All polynucleotide interferon inducers must be doubly-stranded.

2. The 2'-OH group of the ribose moiety is required for activity and is involved in binding to a cellular receptor site.

3. The efficiency of interferon induction is related to the polynucleotides T_m and thus to secondary structure.

4. Human and certain animal sera contain a ribonuclease which can degrade the polynucleotide and thus prevent it from reaching cellular sites of induction in sufficiently high concentration. Since the ribonuclease is an enzyme, such activity should be related to polynucleotide structure.

5. Structural variations may affect the cellular permeability of such materials.

We have prepared and thoroughly characterized a large number of polynucleotide analogs with structural variations which permit examination of the validity of the above hypotheses. These polynucleotides were examined for their interferon-inducing ability by Dr. Sam Baron and Mr. Charles Buckler (NIAID). While none of the polynucleotides had high levels of activity, their activities have allowed us to make several statements regarding the above. Hypothesis 1 was verified. Hypothesis 3 does not hold; poly A·poly U_Z had T_m = 59° yet was considerably less active than Poly A·poly U (T_m = 57°). The necessity of a 2'-OH function (hypothesis 2) was also indicated by our studies, but this specificity does not appear to be absolute. Perhaps most significantly, we have learned that ribonuclease resistance per se (see hypothesis 4) is not a sufficient condition for interferon induction. More recent studies have also indicated that the specificity of the ribonuclease enzyme in human sera is significantly different from that of bovine pancreatic ribonuclease.

This finding, if verified, has substantial importance in the design of interferon inducers for use in humans.

Significance to Biomedical Research and the Program of the Institute: "Fraudulent" nucleosides are, of course, of interest as potential anti-virals, immuno-suppressive or anti-cancer agents. As nucleotides and polynucleotides, such modified nucleosides can be helpful in elucidating the active sites of enzymes involved in nucleic acid metabolism and the physical chemistry of nucleic acids. With respect to interferon, such polynucleotides may lead eventually to the design of a potent inducer which has decreased toxicity which can be used against viral infection and/or cause regression of malignant tumor growth.

Proposed Course: Research on the chemical preparation and characterization of "fraudulent" nucleosides will continue in order to yield new materials with subtle structural modifications. The results of these modifications will be examined at the monomer and polymer level especially with respect to the biological activity of the polynucleotides. Studies have been initiated on the ribonuclease(s) in human sera with the intent of identifying products and the requirements of substrates all to the end of producing effective inducers of interferon.

Honors and Awards: None

Publications:

Kunieda, T. and Witkop, B.: Photo- and stereochemistry of 5,6-methylenepyrimidine nucleosides, bicyclic isomers of thymidine, and 5-methyluridine. J. Am. Chem. Soc. 93: 3478-3487, 1971.

Kunieda, T. and Witkop, B.: Preparation and photochemistry of pyrimidine nucleoside sulfonium ylides. J. Am. Chem. Soc. 93: 3487-3493, 1971.

Kunieda, T. and Witkop, B.: Hydrogenolysis and stereochemistry of photodimers of thymine and thymidine. J. Am. Chem. Soc. 93: 3493-3499, 1971.

Torrence, P. and Witkop, B.: Enzymatic synthesis of polynucleotides containing 5,6-methylene- and 5,6-dihydro pyrimidines. Biochemistry, in press.

Torrence, P., Waters, J., and Witkop, B.: Unexpected conformational stability of poly 2'-azido-2'-deoxyuridylic acid. J. Am. Chem. Soc., in press.

Serial No. NIAMD-LC-23a
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Inhibition of Sodium-Potassium Dependent Adenosine Triphosphatase by Fluorescent Derivatives of Strophanthidin and by Steroidal Alkaloids

Previous Serial Number: SAME

Principal Investigators: F. Oesch and J. Daly

Other Investigators: J. A. Waters and B. Witkop

Cooperating Units: R. W. Albers, NINDS-LNC-

Man Years:

Total: 1
Professional: .5
Others: .5

Project Description:

Objectives: To determine if certain strophanthidin derivatives will exhibit altered fluorescent properties on binding to sodium-potassium activated ATPase.

Methods Employed: Fluorescent properties of strophanthidin derivatives were measured in solvents of different polarity and in the presence of membranal ATPase.

Major Findings: The 3-salicylate and 19-salicylhydrazone derivatives of strophanthidin inhibit sodium-potassium activated ATPase. The former compound exhibits altered fluorescent properties in the presence of the enzyme. The nature of this alteration suggests that the salicylate moiety is present in a relatively nonpolar environment.

Significance to Biomedical Research and the Program of the Institute: Fluorescent inhibitors of Na^+/K^+-dependent ATPase inhibitors should provide powerful tools as fluorescent probes of the enzyme in studying the active site of Na^+/K^+-dependent ATPase and the molecular action of such drugs in their effect on ion transport and on membrane receptors.

Proposed Course: Project terminated.

Honors and Awards: None

Publications:

Oesch, F., Waters, J. A., Daly, J. W., and Witkop, B.: Fluorescent derivatives of strophanthidin: Interaction with sodium-potassium-activated adenosine triphosphatase. J. Med. Chem. In press.

Serial No. NIAMD-LC-23b
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Pyrrole Esters of Various Alkaloids and Steroids

Previous Serial Number: SAME

Principal Investigator: J. A. Waters

Other Investigators: B. Witkop and J. Daly

Cooperating Units: None

Man Years

Total: .3
Professional: .3
Other: 0

This project has been terminated.

Serial No. NIAMD-LC-23c
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Reductive Cleavage of the Oxide Bridge of Veratridine and Related Steroidal Alkaloids

Previous Serial Number: SAME

Principal Investigator: J. A. Waters

Other Investigators: J. Daly and B. Witkop

Cooperating Units: None

Man Years:

Total: 0
Professional: 0
Others: 0

This project has been terminated.

Serial No. NIAMD-LC-24
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on Batrachotoxin, Pumiliotoxin, Histrionicotoxin A and Other Physiologically Active Compounds from Amphibian Skins

Previous Serial Number: NIAMD-LC-27

Principal Investigator: Bernhard Witkop

Other Investigators: J. Daly and D. Johnson

Cooperating Units: Charles W. Myers, American Museum of Natural History, New York, N. Y.;
E. X. Albuquerque, Dept. of Pharmacology, University of New York at Buffalo;
Isabella Karle, U. S. Naval Research Laboratory, Washington, D. C.;
G. Habermehl, Technische Hochschule, Darmstadt, Germany;
T. Tokuyama, Osaka City University, Osaka, Japan;
E. J. Corey, Harvard University;
H. Wehrli, ETH, Zurich.

Man Years:

Total: .3
Professional: .3
Other: 0

Project Description:

Objectives: To elucidate the molecular basis for the pharmacological activity of batrachotoxin, tetrodotoxin, histrionicotoxin and related substances. To isolate and elucidate the structures of other pharmacologically active substances found in skin extracts of neotropical frogs. To explore biosynthetic pathways involved in the formation of these unique substances.

Methods Employed: Sensitive bioassay methods, thin layer, column and gas chromatography have been used in the purification of the active principles, while NMR, mass spectroscopy, x-ray crystallography, microchemical techniques and radiochemical labeling are being utilized for structure elucidation. Isotopic tracer techniques have been used to study biosynthetic pathways.

Major Findings: Investigation of the unique pharmacological properties of batrachotoxin and various analogs has continued. Batrachotoxin appears to increase membrane permeability to sodium ions by interaction with certain membranal proteins. The effect of the drug can be blocked by mild pretreatment

of the membrane with sulfhydryl reagents. The effect of batrachotoxin on spontaneous neuromuscular transmission in Ca^{++}-free media provides evidence for the presence of a mechanism, coupling membrane depolarization to transmitter release, which exhibits a remarkable Q_{10} of >25.

The spiropiperidine alkaloids such as histrionicotoxin isolated from frogs of the genus Dendrobates exhibit a spectrum of interesting pharmacological properties. They first cause a rapid potentiation of the twitch evoked by either direct or indirect stimulation of neuromuscular preparations. They then block the effect of acetylcholine on the muscle endplate receptor. They antagonize the increase in potassium ion conductance associated with action potentials in muscle and nerve, but have no effect on potassium conductance of the resting membrane. After washing to restore the responsiveness of the muscle fiber to acetylcholine, another relatively irreversible effect of such toxins is revealed. Under these conditions they appear to markedly slow the rate of restoration of responsiveness of the acetylcholine receptor after one stimulation. The molecular basis for these properties is under investigation. Slight structural modifications in the toxins result in remarkable shifts in the relative effects on the various components of the cholinergic system. Compounds with almost pure receptor antagonist activity and those with almost pure effects on potassium ion conductances have been revealed.

Comparative chemical taxonomy and pharmacological taxonomy with frogs of this family reveal that batrachotoxin-like compounds and a marked insensitivity to the depolarizing action of batrachotoxin on electrogenic membranes are associated with certain frogs of the genus Phyllobates, while histrionicotoxin-like compounds and a marked insensitivity of the acetylcholine receptor to histrionicotoxin are typically associated with frogs of the genus Dendrobates.

Synthetic Program: Batrachotoxinin A was synthesized in a sequence involving 43 steps by H. Wehrli in the laboratory of Prof. O. Jeger, ETH, Zurich, in March 1972. The conversion to Batrachotoxin is known and has produced the first fully synthetic frog venom.

Pumiliotoxin C is rapidly approaching completion. The synthesis is in progress in the laboratory of Prof. G. Habermehl at the Technische Hochschule in Darmstadt.

Histrionicotoxin's synthesis is well under way in a 14-step sequence in the laboratory of Prof. E. J. Corey, Harvard University.

Significance to Bio-medical Research and the Program of the Institute: The exceptionally high toxicity of batrachotoxin, third among known toxins, its action on nerve preparations and its batrachotoxin properties gave the [illegible] of its structure and correlations of structure with activity a high priority in biomedical research. Other compounds from frog extracts also have physiological activities which warrant their structural and pharmacological investigation. Examples of such compounds occurring in frogs and toads include the pumiliotoxins, histrionicotoxins, samandarine, the bufogenins, the catecholamines, indolealkylamines and histamines, and recently hypotensive polypeptides such as bradykinin and physalaemin. Because of its structural

similarity to bufotenine and lysergic acid diethylamide, a thorough investigation of the properties of dihydrobufotenine is relevant to fundamental aspects of neurochemistry. The novel structure of histrionicotoxin related as it is to that of other alkaloids active in cholinergic mechanisms, warrants a thorough study both of its chemistry and its pharmacology.

Proposed Course of Project: The structures of various toxic alkaloids from frogs of the genera Dendrobates, Colosthetus and Atelopus will be elucidated. The pharmacological activity of batrachotoxin and histrionicotoxin analogs will be studied in detail. Radioactive batrachotoxin, tetrodotoxin and histrionicotoxin will be prepared and used to study their ultrastructural site of action. The unique oxidative cyclization of indolealkylamines to dehydrobufotenine will be investigated and attempts will be made to isolate the enzyme system responsible.

Honors and Awards: Elected to Membership in the "Academia Leopoldina" founded in 1631, as of March 1972.

Publications:

Albuquerque, E. X., Daly, J. W., and Witkop, B.: Batrachotoxin: chemistry and pharmacology. Science 172: 995-1002, 1971.

Daly, J. W. and Witkop, B.: Batrachotoxin, an extremely active cardio- and neurotoxin from the Colombian arrow poison frog, Phyllobates aurotaenia. Clin. Toxicol. 4: 331-342, 1971.

Johnson, D. F., and Daly, J. W.: Biosynthesis of cholesterol and cholesterol acetate in Dendrobatid arrow poison frogs. Biochem. Pharmacol. 20: 2555-2559, 1971.

Witkop, B.: Warum heute noch Naturstoffchemie? Chemie in Unserer Zeit 5: 99-106, 1971.

Daly, J. W., Karle, I., Myers, C. W., Tokuyama, T., Waters, J. A., and Witkop, B.: Histrionicotoxins: Roentgen-ray analysis of the novel allenic and acetylenic spiroalkaloids isolated from a Colombian frog, Dendrobates histrionicus. Proc. Nat. Acad. Sci. U.S. 68: 1870-1875, 1971.

Witkop, B.: Warum heute noch Naturstoffchemie? Kagaku (Chemistry) 26: 1095-1102, 1971.

Albuquerque, E. X., Sasa, M., Avner, B. P., and Daly, J. W.: A possible site of action of batrachotoxin. Nature New Biology 234: 93-94, 1971.

Myers, C. W. and Daly, J. W.: Comment on the proposed designation of a new type-species of Dendrobates. Wagler, 1830. 2.N. (S) 1930. Bulletin of Zoological Nomenclature. In press.

Serial No. NIAMD-LC-25
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Photochemistry of Pharmacodynamic Amines

Previous Serial Number: SAME

Principal Investigator: Bernhard Witkop

Other Investigators: None

Cooperating Units: O. Yonemitsu, Y. Kanaoka and Y. Okuno, University of Hokkaido, Sapporo, Japan
I. L. Karle, U.S. Naval Research Lab., Washington, D.C.

Man Years:

Total: .3
Professional: .3
Others: 0

Project Description:

Objectives: Reactions for the selective modification of simple amino acids in proteins are needed for the study of the composition and function of their active sites. Although extensive interest to develop such reactions has been shown by many investigators, photochemical methods were applied only recently to this problem. Photochemical methods have been elaborated which make it possible to deliver selectively a distinct amount of energy to a system. The photochemical behavior of free and bound aromatic amino acids and the corresponding pharmacodynamic amines and certain of their derivatives therefore have been investigated.

Methods Employed: Ultraviolet irradiation, thin-layer chromatography, silica gel and ion exchange column chromatography, paper chromatography and electrophoresis, UV, IR, NMR and mass spectroscopy.

Major Findings: Irradiation of N-chloroacetyltyrosine in 10% aqueous ethanol gave the ethyl acetate-soluble 7-hydroxy-1,2,4,5-tetrahydro-3H-3-benzazepin-2-one, and two water-soluble dimers in yields of 40 and 12%, respectively. X-ray analyses of single crystals by the symbolic addition procedure established the structure of the 1st dimer as decahydro-7,14a,7a,14-ethanediylidenenaphtho[1,8-de:4,5-d'e']bisazocine-4,6,11,13-(1H,7H,8H,14H)-tetrone, and the second dimer as 4,11-diacetyl-dodecahydro-7H-1,7,8a-ethanylylidene-8,14-methanocyclopropa[1,6]benzo[1,2-d:4,3-d']bisazocine-3,12,15,17-(4H,9H)-tetrone. The first dimer is converted to the second dimer on

irradiation by a bond-switching process which is thought to be initiated by a Norrish-type I cleavage. Mono- and dimethyl homologs of the two dimers were obtained by the irradiation of the corresponding N- or ring-methylated chloroacetyltyramines.

On the basis of a study on solvent effects in the photolysis of N-chloroacetylmescaline a dualistic mechanism, intramolecular electron transfer in [illegible] protic solvents versus intramolecular energy transfer in most organic solvents is proposed.

There is no oxygen effect in the formation of the novel ten-membered lactams from N-chloroacetamides of 3-methoxy- and 3,5-dimethoxyphenethylamines suggestive of a mechanism which consists in hydrogen abstraction and intramolecular recombination within the solvent cage.

Significance to Biomedical Research and the Program of the Institute: The application of photochemical methods to aromatic amino acids and related pharmacodynamic amines and their derivatives affords new heterocyclic systems, new routes to compounds of biological interest and candidates for evaluation of CNS activity.

Proposed Course of Project: Fluorescence quenching and kinetic studies are now in progress in collaboration with Prof. Osamu Yonemitsu and his group at Hokkaido University to determine the mechanism of these fascinating photocyclizations.

Honors and Awards: Invitation to 4th IUPAC Congress on Organic Photochemistry in Baden-Baden, Germany, July 16-22, 1972.

Publications:

Yonemitsu, O., Nakai, H., Okuno, Y., Naruto, S., Hemmi, K., and Witkop, B.: Exciplexes and cage complexes in the photolysis of N-chloroacetylmescaline and other phenethylamines. Photochem. Photobiol. In press.

NIAMD-LC-26
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Service Function, Section on Microanalytical Services and Instrumentation

Previous Serial No.: NIAMD-LC-30

Principal Investigator: David F. Johnson

Other Investigators: P. Parisius, Byron Baer, A. Wong, W. R. Landis, H.Y.C. Yeh, N. Whittaker, B. Miller, H. K. Miller (retired 3/72), A. Wright (retired 3/72).

Cooperating Unit: None

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

The section on Microanalytical Services and Instrumentation was reorganized during the year, following the retirement of the previous Section Chief, Dr. W. C. Alford. Both basic research and service functions are performed by members of the section. A major mission of the organization involves the instrumental and chemical analyses provided to scientists of the Laboratory of Chemistry, NIH, and to a limited extent to personnel of other governmental agencies. Approximately 30 of the more common elements and five or more functional groups are routinely determined on a quantitative basis, using ultramicro, micro, and semi-micro techniques as required. The materials analyzed include organic and inorganic research samples, commercial preparations and various biological specimens. Molecular weights are determined by vapor pressure osmometry in both aqueous and nonaqueous solvents and by mass spectrometry. Instrumental analyses include: mass spectrometry, infrared, nuclear magnetic resonance, ultraviolet, optical rotation, optical rotatory dispersion and flame photometry. Chemical analyses are done by

most of the commonly used techniques including: gravimetric, colorimetric, gasometric, coulometric, potentiometric and volumetric. Assistance in the interpretation of spectra is rendered on request.

During the past year the total number of analyses was about 12,000. These include: carbon-1719, hydrogen-1719, nitrogen-4111 (Kjeldahl-2275, Dumas-1679, Nessler-157), phosphorus-308, sulfur-114, halogens-198, miscellaneous functional groups-271, metals-243, weight loss-95, infrared spectra-89, nuclear magnetic resonance spectra-919 (A60), 400 (HA100) mass spectra-1308. In addition several hundred instrumental analyses have been performed by individual research workers using our equipment in the evening or on weekends.

In the spring of 1971, a Perkin Elmer Model 240 C, H, N Analyzer was installed in the analytical laboratory. It has proven to be a rapid, reliable automation procedure and has resulted in an increased service output and has allowed for a substantial decrease in sample size required. In general, the service function of the analytical laboratory has increased in use over the past year under the capable supervision of Miss Paula Parisius.

The NMR service has been expanded by the addition of a Varian HA-100 spectrometer in addition to the A60. Since October 1971, over 400 spectra have been analyzed and interpreted by a highly qualified specialist, Dr. Herman Yeh, who recently joined this section. Dr. Yeh has also modified the instrument and it now has the capability of doing heteronuclear decoupling and INDOR experiments. Various temperature NMR are also now available. Dr. Yeh is also involved in collaborative studies with individual investigators separate from routine service functions. More routine NMR spectra run on the A60 instrument.

The mass spectrometry service is operating at a high level under the capable specialization of Mr. William Landis. Modification of the inlet source for the instrument is under development to expand the GLC-Mass Spectrometry service. A considerable decrease has occurred in the infrared service and at present, both infrared and NMR (A60) analyses are being performed by one operator, Mr. Noel Whittaker.

A service function of the Section, providing extensive documentation and stocking of organic chemicals, is being continued by Mr. Benjamin Miller. Mr. Miller also maintains a stock of inorganic chemicals, glassware, compressed gases, shop tools and a small glass blowing facility. In addition, as time permits, Mr. Miller is being trained in gas-liquid chromatography in hopes of providing such a service function in the near future.

The Section Chief serves as Project Officer on Contract PH 43-65-32 between NIAMD and the Medical Research Council of England. This contract is for the partial support of a program which provides reference steroid compounds to qualified research workers in the United States as well as to foreign investigators.

No honors or publications.

NIAMD-LC-27
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The study of the Alkaloids from Solanum Congestiflorum (Natri).

Previous Serial No.: None

Principal Investigator: Robert Katz (from 10/10/71)

Other Investigators: David F. Johnson and Yoshio Sato

Cooperative Units: None

Man Years:
Total: .7
Professional: .7
Other: 0

Project Description:

Objectives: Isolation of alkaloid glycosides from Solanum Congestiflorum and identification of their sugar moieties.

Methods Employd and Major Findings: Through repetitive tlc on H_3BO_3 impregnated silica gel plates, three glycosides were purified. Glycosides I and II afforded solacongestidine (as aglycon) by hydrolysis. Glycoside III afforded a different aglycon as yet unidentified. I and II are diosides containing glucose and galactose (1:1), and glucose and xylose (1:1), respectively. III is a trioside containing glucose:xylose:rhamnose (1:1:1). The identification of the sugars involved glc of the butane boronates and peracetates.

Significance: Identification of four alkaloid aglycons from S. Congestiflorum revealed an unusual unsaturated piperidino moiety on C-20, a possible biosynthetical intermediate of Solanum and Veratrum alkaloids. The elucidation of the sugar moieties would help in revealing more of the above mentioned biogenetic correlation.

Proposed course of project: Determination of the sugar sequence and elucidation of the new aglycon.

Publication: None

NIAMD-LC-28
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Steroid Transformations by Tetrahymena Pyriformis

Previous Serial No.: None

Principal Investigator: Nancy S. Lamontagne

Other Investigator: David F. Johnson

Cooperating Unit: Chester E. Holmlured
Department of Chemistry
University of Maryland
College Park, Md.

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objective: The above mentioned cooperating unit has been interested in the growth inhibitory effects of steroids on the protozoa Tetrahymena Pyriformis. Previous studies demonstrated that a variety of steroids of mammalian origin, as well as steroid derivatives such as dialkylaminoethoxy steroids inhibit the growth of T. Pyriformis, and that this growth inhibition is readily reversed by the addition of cholesterol to the medium. The mode of action of these growth inhibitory steroids is not definitely known, but inhibition of NADH oxidation and RNA synthesis, and interference with respiration and formation of lipids have been demonstrated. Of special importance in T. Pyriformis metabolism is the lipid tetrahymenol, a pentacyclic triterpenoid alcohol produced in the organism by a direct, non-oxidative, proton initiated cyclization of squalene. T. pyriformis grown without added steroids produces tetrahymenol as its major unsaponifiable product. Where steroids such as dehydroepiandrosterone are added to the culture an accumulation of squalene occurs with a corresponding decrease in growth rate of the organism. It has been suggested that cholesterol overcomes this

inhibitory effect by performing the role (as yet unknown) of tetrahymenol in the metabolism of T. Pyriformis. Since the focus of attention has always been on the action of steroids on tetrahymena, it was decided that the reverse study, i.e., the effect of tetrahymena on steroids would give additional information on the role of steroid metabolism in Tetrahymena Pyriformis.

Methods Employed and Major Findings: There are no results to report since this project is just being started. Radioactive steroid precursers will be added to T. Pyriformis cultures in the exponential phase of growth. The inoculated culture will be incubated for various lengths of time, then the cells will be separated from the media by centrifugation. Both the cell pellet and media will be extracted, assayed for radioactivity, and chromatographed by column, thin layer, and perhaps paper methods to separate and identify any radioactive metabolic products.

Significance: Unicellular organisms such as the ciliate protozoan Tetrahymena can provide clues for the hormonal action at the cellular and subcellular levels because they have nuclear and other subcellular structures similar to those of higher organisms. T. Pyriformis has been used successfully as a screening organism in the search for hypocholesteremic agents, and is currently being investigated as a possible competitive source of labeled squalene.

Proposed course of project: As described in Methods Employed.

Publications: None

NIAMD-LC-29

1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Chemical structure - biological activity correlations in the enzymatic reactions catechol-O-methyltransferase (COMT) with different substrates.

Previous Serial No.: None

Principal Investigator: Robert Katz (from 10/10/71)

Other Investigators: A. E. Jacobson

Cooperating Units: S. R. Heller (DCRT, HL)

Man Years:
Total: .7
Professional: .7
Other: 0

Project Description:

Objective: To correlate chemical structure of COMT substrates with: a) the products of the enzyme substrate reaction (the meta/para ratio of the O-methyl ethers); b) Km and c) Vm.

Methods Employed and Major Findings: Statistical methods and computer technique are involved in the project. So far, it was found that the meta/para ratio of O-methyl ethers from various COMT substrates can be related to the chemical structure of these substrates before interaction.

Significance: The clarification of the physico-chemical parameters which determine substrate O-methylation can give some insight into the enzyme system.

Proposed course of project: 1) An attempt at graphical interactive molecular orbital (CNDO-2 and INDO) minimal energy calculation via compilation of suitable computer programs. 2) Regression analysis of the minimal energy conformations of substrates compared with biological parameters in the catechol O-methyl-transferase series.

No honors or publications.

NIAMD-LC-30
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: NMR Studies of Mono- and Di-methyl Benzene Oxide-Oxepin Valence Tautomerism

Previous Serial No.: None

Principal Investigator: Herman J. Yeh

Other Investigators: Harry Yagi and Donald Jerina

Cooperating Unit: None

Man Years:
Total: .5
Professional: .5
Other: 0

Project Description:

Objective: Benzene oxide has been shown (by a group of investigators in our laboratory) as an intermediate in enzymatic hydroxylation of benzene. It exists in rapid valence tautomeric equilibrium with oxepin. The equilibrium of this system can be displaced from one extreme to the other by means of substituents or change in solvent. To understand the role of benzene oxide-oxepin system in the enzymatic reaction, it becomes important to understand thermodynamic properties of the system.

Methods Employed and Major Findings: Variable temperature NMR spectroscopic measurements have been employed in the present study. Values of thermodynamic parameters, i.e., $\Delta H^{\ddagger}$, $\Delta G^{\ddagger}$, $\Delta E^{\ddagger}$, $\Delta S^{\ddagger}$ and rate constants for various substituted benzene oxide-oxepin systems are being determined.

Significance: As the importance of benzene oxide chemistry shown in the metabolic reaction, the present study will provide future researchers with useful information.

Proposed course of the project: Various techniques of nuclear magnetic resonance will be engaged in parallel with the research development of the benzene oxide series prepared in this laboratory. NMR examination of substituted benzene oxide-oxepin tautomerism has been successful.

No honors or publications.

NIAMD-LC-31
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Chemical Strucutre - biological activity correlations in the N-substituted morphinan series

Previous Serial No.: None

Principal Investigator: Robert Katz (from 10/10/72)

Other Investigators: A. E. Jacobson

Cooperating Units: None

Man Years:
Total: .7
Professional: .7
Other: 0

Project Description:

Objective: To determine whether chemical structure and biological (analgesic) activity could be related in the N-substituted morphinan series of compounds.

Methods Employed and Major Findings: Statistical methods and computer technique were extensively used. It was found that chemical structure is relatable to the analgesic activity of N-substituted morphinans whether determined by the Eddy hot-plate test or by heat stimulation of the mouse tail, the latter being an essentially time-independent biological screening test.

Significance: The determination of the physical-chemical parameters necessary for analgesic activity can give additional insight into overall class generalizations and perhaps a lead into future synthetic work for drug design.

Proposed course of project: Extension of graphical interactive molecular orbital minimal energy calculation and eventually a regression analysis of the obtained data with the biological parameters correlated above.

No honors or publications.

NIAMD-LC-32
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Stereochemical Course of Nucleophilic Additions to Arene Oxides. An NMR study.

Previous Serial No.: None

Principal Investigator: Herman J. Yeh

Other Investigators: Alan Jeffrey and Donald Jerina

Cooperating Unit: None

Man Years:
Total: .5
Professional: .5
Other: 0

Project Description:

Objective: Arene oxides have been evaluated as causative agents in mutation, carcinogenesis and toxicity via tissue necrosis. The effects are thought to be mediated by nucleophilic reactions with the arene oxides formed during enzymatic hydroxylation of aromatic compounds and subsequent covalent binding to proteins and nucleic acids. Because of its importance to the biochemical research, an investigation of the stereospecificity of nucleophilic addition to arene oxides was suggested.

Methods Employed and Major Findings: Nuclear magnetic resonance has been employed to follow the reaction route. Several simple nucleophiles have been examined with benzene oxides and the following has been observed: methyl lithium adds cis-1,6, sodium sulfide forms a dialkyl sulfide, azide adds trans, and NH_3 or NH_2 do not add.

Significance: This work provides future researchers with useful information of the susceptibility of arene oxides to nucleophilic opening .

Proposed course of the project: This work is part of the benzene oxide series currently under intensive investigation by a group of researchers in this laboratory.

No honors or publication.

NIAMD-LC-33
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Metabolism of C^{14}-Labelled Steroids by Adrenals from Rats with a Mammotropic Pituitary Tumor

Previous Serial No.: None

Principal Investigator: David F. Johnson

Other Investigator: Nancy Lamontagne

Cooperating Unit: Robert W. Bates (NIAMD, LNE)

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objective: Quantitative and kinetic study of steroid biogenesis in rat adrenals subjected to long-term stress by ACTH, growth hormone, and prolactin.

Methods Employed: Radioactive C^{14}-progesterone was incubated with normal rat adrenals and adrenals from rats subjected to long-term stress of tropic hormones of the anterior pituitary. Extracts of the incubates were chromatographed on silicic acid columns by a technique recently developed in the laboratory which allows for separation of adrenocortical and keto-steroids on a single column. Zones of radioactivity from column chromatography are then further characterized and purified by thin-layer techniques utilizing appropriate tritiated metabolites. Endogenous and C^{14} metabolites are then calculated on the basis of C^{14}/H^{3} ratios. Studies in progress are designed to determine quantitative amounts of metabolites produced and kinetic relationships of biosynthesis of normal and stressed adrenals.

Major Findings: Earlier studies in this laboratory demonstrated that the conversion of progesterone was greater by normal rat adrenal tissue than with the adrenal tissue from tumor animals. Also qualitative differences in the steroid metabolites were found in the two groups following C^{14}-progesterone incubation. Studies in progress are in the preliminary stage and no major findings are available at this stage.

Significance: As reported previously, these tumor-bearing rats showed lowered blood cholesterol and splanchnomegaly of the liver, kidney, and heart. The fact that splanchnomegaly did not occur in adrenal-ectomized rats with MfT, and administration of adrenal steroids to adrenalectomized MfT rats did not exhibit marked a splanchnomegaly, suggested that some unknown factor of adrenal origin was the possible causative agent. The present improved technique of column and thin-layer chromatography now in use may lead to identification of the factor involved. Kinetic study may also reveal a contribution of rate in the biosynthetic pathways involved.

Proposed course of project: Continuation of studies in progress.

Publication: None

NIAMD-LC-34
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Study of Solanocapsine and its Derivatives

Previous Serial No.: Same

Principal Investigator: Yoshio Sato

Other Investigators: None

Cooperating Units: Dr. Yao Teh Chang (NIAMD-LBP)
Professor F. G. Standaert, Dept. of Pharmacology, Georgetown University, Washington, D. C.

Man Years:
Total: .5
Professional: .3
Other: .3

Project Description:

This project is terminated.

Publications: None

NIAMD-LC-35
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: A Synthesis of Tomatillidine from Solasodine

Previous Serial No.: Same

Principal Investigator: Genjiro Kusano

Other Investigator: Yoshio Sato

Cooperating Unit: None

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objective: A method using kryptogenin as starting material in the preparation of tomatillidine resulted in a loss of C_{25} configuration in the final step. Solacongestidine and solafloridine, which were obtained through a hydrolysis of their glycosides may be mixtures of their C_{25} stereoisomers. The relationship of tomatillidine, 24-oxosolacongestidine, solacongestidine and verazine must be investigated.

Methods Employed and Major Findings: Starting with solasodine, a 9 step synthesis gave Δ^5-solacongestidine, with retention of configuration at C_{25}. An oxidation of this product is being attempted in order to produce tomatillidine.

Significance: Although the chemistry of tomatillidine, solacongestidine, and solafloridine has been previously investigated in this section, the configuration of C_{25} is still in doubt and some confusion is recognized in this field. An establishment of the stereochemical interrelationship of these several compounds will result in a further advancement in the chemistry of steroidal alkaloids.

Proposed course of Project: A hydrolysis condition to obtain stereochemically pure solacongestidine and solafloridine should be sought. A treatment with $NaIO_4$ is recommended.

Honors: Dr. Y. Sato was invited to give a lecture at the 1971 International Symposium of Heterocyclic Compound held in Sendai, Japan.

Publications: Sato, Y. and Nagai, M.: Degradation of Solasodine. J. Org. Chem., in press 1972.

NIAMD-LC-36
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Intramolecular Interaction of (5-Methylimidazole-4yl) Dimethyl Butyric Acid and Its Analogs. NMR studies of the model compounds.

Previous Serial No.: None

Principal Investigator: Herman Yeh

Other Investigator: Y. Kikugawa, and L. Cohen

Cooperating Unit: J. Cohen (DCRT)

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objective: Considerable evidence exists to indicate that the imidazole side chain of histidine is involved in a catalytic action of esterases. This is thought that imidazole act intramolecularly as a general base catalyst in the hydrolysis of activated esters. To understand its precise role in the hydrolysis reaction, the study of physical properties of these model compounds (prepared in this laboratory) is necessary.

Methods Employed and Major Findings: NMR technique is being used to examine the intramolecular effects between the carboxyl and imidazole groups. pk_A values, reactivities and other physical properties of these model compounds will be determined.

Significance: Results of this study will permit more thorough interpretation of biological tests and will indicate additional biological experiments which should be performed.

Proposed course of the project: NMR examination of this system has been successful and the study will be extended in order to examine the enzyme system.

No honors or publications.

NIAMD-LC-37
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The study of the Constituents of Solanum Xanthocarpum

Previous Serial No.: Same

Principal Investigator: Genjiro Kusano and Robert Katz

Other Investigator: Yoshio Sato and David F. Johnson

Cooperating Unit: Malcolm J. Thompson and William E. Robbins, Insect Physiology Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objective: To isolate and identify the highly toxic component effecting the housefly larvae found in the extracts of Solanum xanthocarpum.

Methods Employed and Major Findings: A systematic chromatographic investigation of ligroin and alcoholic extracts from S. xanthocarpum has resulted in 9 new steroidal compounds together with 11 known neutral steroids. The structures of the new compounds were determined through interconversion from carpesterol. Although solamargine, a steroidal alkaloid glycoside, has some larvaecidal activity, the mixture of solamargine and other glycosides is more active. Because three other glycosides, β-solamargine, solasonine and an unidentified glycoside are found to be far less active, a possible synergistic effect is suggested.

Significance: Some biologically active compounds affecting insect physiology are of considerable significance in the control of these insects and in the general field of insect biochemistry. Furthermore, present research on the constituents of this plant suggests an important role of esterification with aromatic acids and glycoside linkage formation in the early stage of the biogenesis of the compounds.

Proposed course of project: A quantitative bioassay will be attempted in

order to support a kind of synergism in biological effect by solarmargine and other glycosides on insects. Some chemical and biochemical investigation will be pursued to elucidate the role of esterification with aromatic acids.

Publications: Kusano, G., Beisler, J., and Sato, Y.: The Constituents of Solanum Xanthocarpum. Phytochemistry, in press 1972.

NIAMD-LC-38
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Synthesis of Solaphyllidine

Previous Serial No.: Same

Principal Investigator: Genjiro Kusano

Other Investigator: Yoshio Sato

Cooperating Units: None

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objective: To synthesize solaphyllidine (22,26-epimino-5α-cholestan-4-one-3β,16α,23-triol 16-monoacetate), a new steroidal alkaloid isolated from Solanum hypomalacophyllum Bitter, utilizing solasodine as starting material.

Methods Employed and Major Findings: 4-Deoxo-Δ^5-solaphyllidine, prepared synthetically according to modified methods adopted in the synthesis of solafloridine and 4-deoxosolaphyllidine (see NIAMD-LC-38, 1970-1971), was oxidized to dihydro-Δ^5-solaphyllidine with SeO_2 and the latter was oxidized to Δ^5-solaphyllidine with active MnO_2. A selective reduction of the double bond at C_5 is being attempted to convert Δ^5-solaphyllidine to solaphyllidine.

Significance: Solaphyllidine possesses several functional groups on a steroidal skeleton that suggests a possibility of pharmacological activity. Its synthesis, including side reaction products, therefore, provides some data on the relationship between structure and biological activity of steroidal alkaloids.

Proposed Course of Project: Biological assay of certain compounds will be continued.

Publications: None

NIAMD-LC-39
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: 1. Conversion of Solaphyllidine to Solanocapsine
2. Steroidal Alkaloids - Isomerism of Solasodine Formates

Previous Serial No.: 1. same
2. same

Principal Investigator: 1. Genjiro Kusano
2. Genjiro Kusano

Other Investigator: 1. Yoshio Sato
2. Yoshio Sato

Cooperating Units: 1. Professor Alfredo Usubillaga, Instituto de Investigacion Quimica, Universidad de Los Andes, Facultad de Farmacia, Merida, Venezuela
2. Edwin D. Becker, NIAMD-LPB

Project Description:

1. This project is terminated.
2. This project is now NIAMD-LC-41

NIAMD-LC-40
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Metabolism of C^{14}-Labelled Steroids by Adrenals from Pseudohermaphrodite Rats

Previous Serial No.: Same

Principal Investigator: David F. Johnson

Other Investigator: Nancy Lamontagne

Cooperating Unit: C. Wayne Bardin, formerly Endocrinology, NCI; present Pennsylvania State Medical School, Hershey, Penn.

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objective: The above mentioned cooperating unit has been studying an unique colony of rats in which a genetic defect, carried by normal females, is passed on to half the genetic males. Half the genetic males are pseudohermaphrodites. These pseudos have both male and female characteristics, although the tests are internal. It has been demonstrated that they have a defect of testosterone synthesis, suggesting a general deficiency of the 17-α-hydroxylase enzyme systems may be involved. The present study is designed to test this hypothesis in the adrenal gland of these animals.

Methods Employed and Major Findings: In previous studies, radioactive progesterone was incubated with normal rat adrenals (male and female) and adrenals from pseudohermaphrodite rats. Extracts of the aqueous incubation media and intact tissue were separately analyzed by column and thin-layer techniques. Isolated radioactive metabolites were then characterized by comparison with known steroid standards. No differences were found in the tissue extracts on comparison of pseudo with normal male and female rat

adrenals. Significant differences in metabolites, both quantitatively and qualitatively were observed. Of particular significance in the pseudo rat adrenal was the unusual production of 4-androstene-3,17-dione, a direct precursor of testosterone. Studies in progress are being directed to a detailed investigation of the production of intermediates in testosterone production from endogenous non-radioactive and radioactive progesterone. Kinetic studies as a part of this investigation may reveal differences in production rate as a hunting factor in the two classes of adrenals.

Significance: The preliminary findings of this study suggest that further experiments may lead to a better understanding of the interrelated biosynthesis of adrenocortical steroids, androgens, and estrogens. These animals are especially suited to such study since the defective testosterone synthesis has been well established in testicular tissue and our preliminary findings are strongly indicative of a defective 17-β-hydroxysteroid dehydrogenase enzyme system in the adrenal tissue.

Proposed course of project: Continued study of the biosynthetic pathways involved in normal and pseudo rat adrenal, with particular emphasis on the quantitative and kinetic production of selected steroids.

Publications: None

NIAMD-LC-41
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Pyramidal Isomerism of Solasodine and its Derivatives

Previous Serial No.: None

Principal Investigator: Genjiro Kusano

Other Investigators: Herman J.C. Yeh and Yoshio Sato

Cooperating Unit: None

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objective: Solasodine shows some chemical properties different from its C_{22}, C_{25} isomer, tomatidinol. This difference may result from the fact solasodine has a specific pyramidal isomerism. This phenomenon may occur in other heterocyclic compounds, and general methods of differentiating these pyramidal isomers would be useful.

Methods Employed and Major Findings: Solasodine gave two kinds of N-formates. One of them afforded 16-O,N-diformate by opening ring E under drastic formylation conditions, while the other had no change, suggesting that the two N-formates are pyramidal isomers and not cis-trans isomers of the N-formyl group. Other solasodine and tomatidinol N-derivatives were prepared and NMR spectra were measured to support the hypothesis of pyramidal isomerism in solasodine and its derivatives.

Significance: Pyramidal isomerism may occur in many other heterocyclic compounds, some of which are used as drugs. This kind of isomerism may afford different biomedical activity, therefore, adequate methods of differentiating these isomers would be useful in such studies.

Proposed course of project: Additional solasodine and tomatidinol N-derivatives will be prepared and the NMR spectra will be measured under various temperature conditions in a further study of pyramidal isomerism.

Publications: None

NIAMD-LC-42
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Toxicity Studies of Solanum Bulbocastanum

Previous Serial No.: Same

Principal Investigator: David F. Johnson

Other Investigators: Yoshio Sato (deceased)

Cooperating Units: Potato Introduction Project
USDA Agricultural Research Service
Department of Horticulture
University of Wisconsin
Madison, Wisconsin

Man Years:
Total: 0
Professional: 0
Other: 0

Project Description:

This project still held in abeyance pending repeat of isolation and testing if plants become available in a growing season under ideal conditions of growth.

Publication: None

NIAMD-LC-43
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Biosynthesis of Stigmasten-3β-ol and 3′,5′-Cyclic AMP by the Slime Mold (Dictyostelium discoideum)

Previous Serial No.: Same

Principal Investigator: David F. Johnson

Other Investigators: None

Cooperating Unit: Micah Krichensky, (D:IR)

Man Years:
Total: 0
Professional: 0
Other: 0

Project Description:

This project held in abeyance pending completion and write-up by cooperating unit.

Publication: None

NIAMD-LC-44
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Column Chromatography of Adrenocorticol Steroids and Ketosteroids by Gradient Elution

Previous Serial No.: same

Principal Investigator: David F. Johnson

Other Investigator: Nancy Lamontagne

Cooperating Unit: Grant C. Riggle, Instrument Engineering and Development Branch, DRS, NIH

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

This project is now terminated as study of methodology involved in gradient elution of steroids. The techniques and apparatus developed are now being used extensively in other projects involving chromatographic separation of complex steroid mixtures.

NIAMD-LC-45
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Study of the Photochemical Reactions of Certain Derivatives of Indole and Related Heterocyclic and Carbocyclic Compounds.

Previous Serial No.: NIAMD-LC-44

Principal Investigator: Calvin M. Foltz

Other Investigators: None

Cooperating Units: None

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objective: To develop organic chemical and photochemical methods of utility in the study of biological and medical problems and to synthesize organic compounds of biological and medical interest. To study the mechanism of the photocyclodehydrohalogenation reaction produced by uv irradiation of N-chloroacetyltryptamine and related compounds and to study the scope of its applicability to other heterocyclic and non-heterocyclic systems. To study the scope and mechanism of the rearrangement which has been found to take place on uv irradiation of 3-(3'-chloro-2'-oxopropyl)indoline-2-one. To study the photo-Fries rearrangement of esters, particularly of halo-acids, with a view to developing methods for modifying and synthesizing compounds of potential biological and medical interest.

Methods Employed: Standard organic chemical techniques; irradiation of solutions of organic compounds with ultraviolet radiation generated by mercury-vapor lamps; column, gas, and thin layer chromatography; and ultraviolet, visible, infrared, nuclear magnetic resonance, and mass spectroscopy.

Major Findings: Photolysis of N-chloroacetyltryptamine, N-chloroacetyl-3-(3'-aminopropyl)-indole, and N-3-(4'-aminobutyl)indole with mercury-vapor lamps results in the formation of high-melting dehydrohalogenation products in yields of 50%, 30%, and 20%, respectively. The products are thought to be

the tricyclic lactams resulting from cyclization in the 4-position of the indole moiety: 6-oxo-3,4,6,7-tetrahydro-1H,5H-azocin[4,5,6-c,d]indole, 7-oxo-3,4,5,6,7,8-hexahydro-1H-azonin[6,5,4-c,d]indole and 8-oxo-3,4,5,6,8,9-hexahydro-1H,7H-azecin[6,5,4-c,d]indole, respectively. However, the ir spectra do not confirm these structural assignments. Photodehydrohalogenation products have been prepared for reduction to the amine with diborane. The improved solubilities of the amines will permit careful nmr studies which should make possible final structural assignments for these compounds.

As part of the study of this photodehydrohalogenation reaction N-chloroacetyl-2-(α-naphthyl)ethylamine was prepared as a prototype of polycyclic aromatic systems. Irradiation with a mercury-vapor lamp resulted in photocyclization with the formation of the tricyclic lactam, 2-oxo-2,3,4,5-tetrahydro-1H-naphth[1,8-de]azocine, in 47% yield. N-Choroacetyl derivatives of compounds such as aniline, benzylamine, β-phenylethylamine, 1-aminomethylnaphthalene, and α- and β-naphthylamines also have been prepared and phototitrations carried out. The utility of the automatic photititration technique was discovered in the course of study of photodehydrohalogenations and already has been applied to several series of compounds. The kinetic data obtained in this way provides useful information on the course and mechanism of such reactions. Further refinements in the technique to extend its utility are being made. Typical results are the following: N-chloroacetyltryptamine $t_{1/2}$=1.5 min (Vycor filter); N-chloroacetyl-4-(3'-indolyl)butylamine $t_{1/2}$=1.4 min (Vycor filter); N-chloroacetyl-m-tyramine $t_{1/2}$=1.2 min (Vycor filter); N-chloroacetylmescaline $t_{1/2}$=11.6 min (Vycor filter); N-chloroacetyldopamine $t_{1/2}$=2.4 min (Vycor filter); N-chloroacetylnormescaline $t_{1/2}$=17.5 min (Vycor filter); N-choroacetyltryptamine $t_{1/2}$=0.6 min (no filter); N-chloroacetylhomotryptamine $t_{1/2}$=0.6 min (no filter); N-β-chloropropionyltryptamine $t_{1/2}$= 3.5 min (no filter); N-α-chlorobutyryltryptamine $t_{1/2}$=62 min (no filter). Several years ago in the course of studies of intermolecular photodehydrohalogenation reactions, anomalous phototitration results were obtained with phenyl chloroacetate as one of the reactants. A literature search revealed that phenyl chloroacetate apparently undergoes a photo-induced Fries rearrangement followed by a cyclodehydrohalogenation reaction. These reactions may afford a mild specific method for modifying certain compounds of biological or medical interest or for synthesizing compounds of special interest. Studies of this reaction are in progress.

Significance: New compounds produced in this work are of biological and medical interest. The organic chemical and photochemical reactions being developed can be applied to the synthesis of other compounds of biological and medical interest, to the modification of compounds of biological and medical interest, and to the development of methods for use in the study of biological and medical problems.

Proposed course of project: To continue to study the mechanisms of the reactions described in this report and to determine the extent to which they can be applied to related compounds, to related types of compounds and particularly to compounds of biological interest. To apply the methods developed to the synthesis of compounds of particular biological or medical interest. To develop and study other photochemical and organic chemical reactions and techniques with potential utility for the synthesis, modification, characterization, and study of molecules of biological and medical interest. To develop chemical methods which can be applied in the study of biological and medical problems.

Honors and awards: None

Publications: None

NIAMD-LC-46
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Structure Determination of N,N,N'-Trimethylsolanocapsine by X-ray Crystallography

Previous Serial No.: same

Principal Investigator: John A. Beisler

Other Investigators: Y. Sato (NIAMD-LC) and J. V. Silverton (NHLI-LC)

Cooperating Unit: None

Man Years:
Total: .3
Professional: .3
Other: 0

Project Description:

This project is terminated.

NIAMD-LC-47
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Structure Determination of Cedrone by X-ray Crystallography

Previous Serial No.: same

Principal Investigator: John A. Beisler

Other Investigators: H. M. Fales and J. V. Silverton (NHLI-LC)

Cooperating Unit: None

Man Years:
Total: 0
Professional: 0
Other: 0

Project Description:

This project is terminated.

NIAMD-LC-48
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NHI
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Degradation and Structural Elucidation of the Triterpenoid, Carpesterol.

Previous Serial No.: same

Principal Investigator: John A. Beisler

Other Investigator: Yoshio Sato

Cooperating Unit: J. V. Silverton, Georgetown University

Man Years:
Total: .3
Professional: .3
Other: 0

Project Description:

This project is terminated.

NIAMD-LC-49
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The von Braun Degradation of Demissidine

Previous Serial No.: same

Principal Investigator: John A. Beisler

Other Investigator: Yoshio Sato

Cooperating Unit: None

Man Years:
Total: .2
Professional: .2
Other: 0

Project Description:

This project is terminated.

NIAMD-LC-50
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Synthesis of Camptothecin Analogs

Previous Serial No.: same

Principal Investigator: John A. Beisler

Other Investigator: E. M. Fry

Cooperating Unit: CCNSC

Man Years:
Total: .3
Professional: .3
Other: 0

Project Description:

This project is terminated.

NIAMD-LC-51
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Nuclear Magnetic Resonance Spectra of Codeine and Isocodeine Derivatives. The Magnetic Anisotropy of a Spiro Oxirane Ring

Previous Serial No.: None

Principal Investigator: Herman Yeh

Other Investigator: Arthur Jacobson and Lewis J. Sargent

Cooperating Units: None

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objective: Structural determinations of a spiro oxirane (1) derivative of codeinone, codeine (2), isocodeine (3), 6-methylcodeine (4), and 6-methylisocodeine (5).

Methods Employed and Major Findings: NOE and double resonance experiments have been used to confirm the structures of these titled compounds.

Significance: NMR examinations of these compounds have been successful. This study provides researchers with useful information.

Proposed course of the project: This work has been completed.

Publication: Jacobson, A. E., Yeh, H.J.C., and Sargent, L. J.: Nuclear Magnetic Resonance Spectra of Codeine and Isocodeine derivatives. The Magnetic Anisotropy of a Spiro Oxirane Ring. Org. Magn. Resonance, in press 1972.

NIAMD-LC-52
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: ^{1}H and ^{19}F NMR Studies of Ring Flourinated Imidazole Derivatives

Previous Serial No.: None

Principal Investigator: Herman Yeh

Other Investigators: Ken Kirk and Louis Cohen

Cooperating Units: Jack Cohen (DCRT)

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objective: Ring flourinated imidazole derivatives (e.g., fluorohistidine and fluorohistamine) are being studied in this laboratory as enzyme inhibitors and as potential drugs. The studies of the chemico-physical properties of these compounds and their interaction with enzymes are required to understand biochemical interactions.

Methods Employed and Major Findings: ^{1}H and ^{19}F NMR experiments and molecular orbital calculations have been used in this work. Values of chemical shifts, coupling constants and MO calculations will be used to determine the electronic distribution in this system. pK_A values of these compounds have been determined (by pH titration followed by NMR observation) to be $2.46 \pm .02$ $2.52 \pm .02$ and $1.83 \pm .02$ for 2-fluoro-imidazole, 4-fluoro-imidazole and 4-fluoro-histidine, respectively.

Significance: Results of physical-chemical studies on these compounds will permit more thorough interpretation of biological tests and will indicate additional biological experiments which should be performed.

Proposed course of the project: Future plans include incorporation of ring fluorinated histidine into protein structure of enzymes and NMR investigation of the fluorinated enzyme during interaction with substrates and inhibitors.

No honors or publications.

NIAMD-LC-53
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: A synthesis of ecdysone-like compounds from carpesterol

Previous Serial No.: None

Principal Investigator: Genjiro Kusano

Other Investigator: Yoshio Sato

Cooperating Unit: William E. Robbins
Insect Physiology Laboratory
Agricultural Research Service
U. S. Department of Agriculture
Beltsville, Maryland 20705

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objective: New ecdysone-like compounds are wanted for moulting hormone activity, antiecdysone activity or chemosterilant activity on insects. Several new ecdysone-like compounds are being synthesized from carpesterol in a few steps.

Methods Employed and Major Findings: Starting from carpesterol, 4α-methyl-(24R)-ethyl-coprost-6-on-7-ene-3β,14d,22R-triol, 4α-methyl-(24R)-ethyl-coprost-6,22-dion-7-ene-2β,3β,14α-triol and 4α-methyl-(24R)-ethyl coprost-6-on-7-ene-2β,3β,14α,22R-tetraol were synthesized by a 6-step reaction series.

Significance: In addition to the effects on insects, some ecdysones are reported to have a stimulatory effect on protein synthesis in higher animals (S. Okui, T. Otaka, M. Uchiyama, T. Takemoto, H. Hikino, S. Ogawa, and N. Nishimoto: Chem. Pharm. Bull., 16, 384-387, 1968). These new ecdysone-like compounds may have some activity.

Proposed course of Project: A biological assay is in progress on these new ecdysone-like compounds.

Publications: None

Serial No. NIAMD-LC-54
1. Chemistry
2. Pharmacodynamics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on Pharmacodynamic Amines and Enzymes Involved in Their Metabolism.

Previous Serial Number: NIAMD-LC-32

Principal Investigator: C. R. Creveling

Other Investigators: J. Daly and J. Lundstrom

Cooperating Units: J. Milstien, NIAMD:LPB
L. Goldberg, Emory University, Atlanta, Georgia
E. Costa, St. Elizabeths Hospital, NIMH
K. Fuxe, Karolinska Institute, Stockholm, Sweden
W. Lutz, Manchester College, North Manchester, Indiana

Man Years

Total: 3.7
Professional: 2.3
Other: 1.5

Project Description:

Objectives: To study the basic chemistry and biochemistry of the catecholamines, phenolic amines, indolealkylamines, imidazolealkylamines and their precursors and transformation products. To develop new techniques for the investigation of transport metabolism and binding phenomena as related to these amines and their interactions with various drugs. To isolate and purify enzymes operative in the biosynthesis and metabolism of biogenic amines. To determine the physical properties of these enzymes and the effect of pH, temperature, substrate specificity, reversible and irreversible inhibitors and the ionic composition of the incubation media. To develop assays and to apply these enzymatic assays to determine specific biogenic amines and to study the distribution and variation of enzyme levels. To develop new pharmacologically active agents for the study of the biological parameters that affect biogenic amines.

Methods Employed: Drugs and substrates labeled with tritium, deuterium or C^{14} have been used to study the metabolic fate of endogenous amines and amino acids and the underlying mechanisms of uptake, release and metabolism of these compounds. Standard methods of enzyme purification have been used including: differential centrifugation, density gradient centrifugation, gel filtration, electrophoresis, standard column chromatography.

Major Findings: The 3-thio analog of dopamine was a potent irreversible inhibitor of catechol-O-methyltransferase in vitro but not in vivo. Inhibition was caused by the formation of a disulfide bridge to a sulfhydryl group in the active site of the enzyme and could readily be reversed with dithiothreitol or glutathione. In addition, the 3-thio analog of dopamine was i) a substrate of mitochondrial monoamine oxidase and a competitive inhibitor with tyramine as substrate, ii) a potent noncompetitive inhibitor of dopamine-α-hydroxylase, iii) a noncompetitive reversible inhibitor of phenethanolamine-N-methyltransferase. The compound did not cross the blood brain barrier, caused significant release of cardiac norepinephrine at high dosages and was rapidly eliminated in free form and as conjugates of itself and of the alcohol and acid formed by deamination.

Substantial improvement in the isolation and purification of catechol-O-methyltransferase was accomplished by use of affinose column chromatography. A preparation of enzyme with 80% purity was attained. This material was incubated with double-labeled radioactive S-adenosylmethionine in the absence of catecholic substrate. The enzyme on separation from unreacted S-adenosyl methionine by Sephadex chromatography was found to contain radioactive methyl groups which appeared to be partially transferred to catechols on subsequent incubation.

The effects of all the trihydroxyphenethylamines and certain tetrahydroxyphenethylamines on the in vivo uptake and release of radioactive norepinephrine in mouse have been measured. 2,4,5-Trihydroxyphenethylamine (6-hydroxydopamine) and the 2,3,5-analog cause both initial release of norepinephrine and long term destruction of adrenergic binding sites. The 3,4,5 and 2,3,4 analogues are active only in terms of causing initial release, while the 2,4,6 and 2,3,6 isomers are relatively inactive in this system. The technique of labeling with [^{3}H]-norepinephrine has also been adopted for studies on uptake, storage and release in both arteries and veins.

5,6-Dihydroxytryptamine was prepared and found to cause selective depletion of serotonin in the central nervous system after intraventricular and intracerebral administration of the drug. Initially, 5,6-dihydroxytryptamine causes some release of norepinephrine, but its long term effects appear due to selective destruction of serotonergic systems similar to the selective destruction of adrenergic systems evoked by 6-hydroxydopamine.

Significance to Biomedical Research and the Program of the Institute: The biosynthesis and metabolism of biogenic amines are of fundamental im-

portance to the fields of neurochemistry, pharmacology and have a direct bearing on the understanding of the functioning of the cardiovascular system.

Proposed Course of Project: The binding and release of norepinephrine as influenced by drugs will be explored further, as will interactions of certain amines and drugs at storage and receptor sites. Studies on active site labeling and the primary structure of catechol-O-methyltransferase will be continued. Studies on inhibitors of tyrosine hydroxylase, phenylalanine hydroxylase, dopamine-β-hydroxylase and catechol-O-methyltransferase will be continued. Attempts will be made to prepare antibodies to catechol-O-methyltransferase. The possible significance of false transmitters for histamine and serotonin will be explored. An attempt to rationalize the kinetic interactions of COMT, substrate, metal ion and S-adenosylmethionine in terms of a reaction mechanism and the formation of an active methyl-enzyme intermediate is in progress. The interactions of thio analogs of phenolic and catecholic biogenic amines with a variety of enzymes and biological systems will be explored in greater detail.

Honors and Awards: None

Publications:

Lutz, W. B., Creveling, C. R., Daly, J. W., Witkop, B. and Goldberg, L. Sulfur Analogs of dopamine and norepinephrine: Inhibition of Catechol-O-Methyltransferase. *J. Med. Chem.* In press.

Creveling, C. R., Morris, N., Shimizu, H., Ong, H. H., and Daly, J.: Catechol-O-Methyltransferase. IV: Factors Affecting *Meta*- and *Para*-Methylation of Substituted Catechols. *Mol. Pharmacol.* In press.

Serial No. NIAMD-LC-55
1. Chemistry
2. Pharmacodynamics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Role of Cyclic Adenosine Monophosphate in the Central Nervous System.

Previous Serial Number: NIAMD-LC-31

Principal Investigator: John Daly

Other Investigators: M. Huang, J. Schultz

Cooperating Units: F. Bloom, St. Elizabeths Hospital, NIMH
E. Gruenstein, NIAMD:CE
B. Hamprecht, NHLI:LBG
H. Shimizu, Kumamoto University, Kumamoto, Japan

Man Years

Total: 3.7
Professional: 1.3
Other: 2.5

Project Description:

Objectives: To determine the factors that govern the formation and metabolism of cyclic AMP in the central nervous system. To investigate the possible role of cyclic AMP in regulating biochemical reactions in brain tissue.

Methods Employed: Isotopically labeled compounds have been used to study the formation and breakdown of adenine nucleotides in brain slices in cultured tumor cells and with partially purified enzymes.

Major Findings: Cyclic AMP accumulates in guinea pig cerebral cortical slices during incubations with histamine, norepinephrine, serotonin, adenosine, veratridine and combinations of these agents. The increases in endogenous levels of cyclic AMP and the increases in [^{14}C] cyclic AMP derived from intracellular nucleotides are quite similar but follow slightly different time courses. An accumulation of cyclic AMP in response to histamine or a histamine-norepinephrine combination can be

elicited only one time, except in the presence of adenosine. The phosphodiesterase inhibitors, papaverine and isobutylmethylxanthine, are much more effective than theophylline in enhancing the amine-stimulated increase in cyclic AMP in brain slices. None of the phosphodiesterase inhibitors potentiated the effect of adenosine or adenosine-amine combinations on cyclic AMP levels. Comparison of "specific activity" of adenine nucleotides and cyclic AMP suggests that [^{14}C] adenine is incorporated into morphological compartments of adenine nucleotides which represent less than 30% of the total nucleotides of the slice. Localization of these compartments by combination electron microscopy-radioautography has as yet been unsuccessful.

The results of studies on the effect of adenosine on accumulation of cyclic AMP in the presence of adenosine antagonists or of compounds which prevent the uptake of adenosine into brain slices suggest that the site of action of adenosine is extracellular.

Accumulations of cyclic AMP elicited in brain slices by tricyclic psychotropic drugs such as imipramine and chlorpromazine are mediated by a prior decrease in ATP levels and resultant "release" of adenosine. Tissue levels of ATP decrease presumably due to the interference with mitochondrial oxidative phosphorylation caused by this class of drugs.

Rat glioma cells incorporate [^{14}C] adenine into nucleotides that subsequently can be converted to cyclic AMP in the presence of norepinephrine. The phosphodiesterase inhibitors, papaverine and isobutylxanthine, effectively enhance the stimulatory effect of norepinephrine. Elevated levels of cyclic AMP in these cultured cells elicites an apparent increase in phosphodiesterase activity. In contrast to results with brain slices, repetitive accumulations of cyclic AMP can be elicited during a series of restimulations of glial cells with norepinephrine. In addition, a large portion of the [^{14}C] adenine is incorporated in glioma cells into intracellular compounds that do not serve as precursors of [^{14}C] cyclic AMP.

The order of relative activity of various local anesthetics as antagonists of the veratridine-elicited accumulation of cyclic AMP in brain slices was consonant with the order of their relative toxicity in clinical use for spinal anesthesia.

Significance to Biomedical Research and the Program of the Institute: The key role of adenyl cyclase and cyclic AMP in regulating cellular activity in response to external stimuli in many biological systems make elucidation of their role in brain of fundamental importance to an understanding of function in this organ.

Proposed Course of Project: The morphological location of the labeled pools of adenine nucleotides will be determined. The role of phosphoribosyl transferase and adenosine kinase in maintaining this pool will be explored. The turnover of cyclic AMP formed after treatment with

amines, adenosine or depolarizing agents will be measured. Possible secondary effects of elevated cyclic AMP levels in brain tissue will be sought. The in vivo effects of drugs on the responsiveness of the cyclic AMP system will be explored. Attempts to label the precursor pool with fluorescent and radioactive adenine analogs will be made. The cyclic AMP-generating system will be investigated in rabbit superior cervical ganglion and in mouse and rat cerebellum and cerebral cortex.

Honors and awards: None

Publications:

Schultz, J., Hamprecht, B., and Daly, J. W.: Accumulation of Adenosine 3',5': Cyclic Monophosphate in Clonal Glial Cells: Labeling of Intracellular Adenine Nucleotides with Radioactive Adenine. *Proc. Nat. Acad. Sci. USA*. In press.

Huang, M., and Daly, J. W.: The Accumulation of Cyclic Adenosine Monophosphate in Incubated Slices of Brain Tissue. I. Structure-Activity Relationships of Agonists and Antagonists of Biogenic Amines and of Tricyclic Tranquilizers and Antidepressants. *J. Med. Chem.* In press.

Huang, M., Shimizu, H., and Daly, J. W.: The Accumulation of Cyclic Adenosine Monophosphate in Incubated Slices of Brain Tissue. II. The Effects of Depolarizing Agents, Membrane Stabilizers, Phosphodiesterase Inhibitors and Adenosine Analogs. *J. Med. Chem.* In press.

Daly, J. W.: Accumulation of Cyclic AMP in Tissue Slices and Intact Cells. In Chasin M. (Ed.): *Methods Used in Cyclic AMP Research*. New York, Marcel Dekker, vol. V. In press.

Daly, J. W., Huang, M., and Shimizu, H.: Regulation of Cyclic AMP Levels in Brain Tissue. In Greengard, P., Robison, G. A., and Paoletti, R. (Eds.): *Advances in Cyclic Nucleotide Research*. New York, Raven Press, vol. I. In press.

Serial No. NIAMD-LC-56
1. Chemistry
2. Pharmacodynamics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Mechanism of Enzymatic Hydroxylation and The Role of Reactive Intermediates in Drug Metabolism.

Previous Serial Number: NIAMD-LC-33

Principal Investigator: Donald M. Jerina

Other Investigators: J. Daly, H. Yagi, A. Jeffrey, F. Oesch, H. Yeh, and B. Witkop.

Cooperating Units: D. Boyd, Univ. Belfast, Belfast, Ireland
H. Ziffer, NIAMD:LPB
D. Reed, Oregon State Univ., Corvallis, Oregon
O. Chapman, Iowa State Univ., Ames, Iowa
J. Rice, NCI:ET
A. Y. H. Lu, Hoffmann-LaRoche, Nutley, New Jersey
D. W. Nebert, NICHHD:LBS
J. P. Ferris, Rensselaer Institute, Troy, New York
T. C. Bruice, Univ. of California, Santa Barbara, Calif.
G. Berchtold, Massachusetts Institute of Technology, Cambridge, Massachusetts
E. D. Becker, NIAMD:LPB

Man Years

Total: 2.7
Professional: 2.5
Other: 0.3

Project Description:

Objectives: To study the basic mechanism of oxygen activation and enzymatic incorporation into organ substrates. To explore enzymatic and non-enzymatic reactions of labile intermediates such as epoxides and cyclic peroxides.

Methods Employed: Isotopically labeled substrates, water and oxygen gas, have been used to study the oxidation of organic compounds with a variety of enzymes and the subsequent metabolism of labile intermediates. Nuclear magnetic resonance spectroscopy, mass spectrometry, x-ray crystalography, and other physico-chemical techniques have been employed frequently in these studies.

Major Findings: The differential inhibition by an epoxide hydrase inhibitor of the formation of dihydrodiol from either naphthlene or naphthalene oxide in liver microsomes gives evidence for the presence of tightly-coupled membranal monooxygenase-hydrase systems which convert arenes to dihydrodiols. The intermediate arene oxide does not in this case fully equilibrate with exogenous arene oxide.

Cytochrome P-450 and P-448 fractions isolated from rat liver contain high levels of epoxide hydrase. A reconstituted P-450 system metabolizes naphthalene to equal amounts of naphthol and dihydrodiol via the intermediate naphthalene oxide. The monooxygenase and hydrase enzymes appear more closely associated in the P-448 system, where the predominant product from naphthalene is the dihydrodiol.

Levels of epoxide hydrase in inbred and hybrid C57 BL/6N and DBA/2N mice are similar and in contrast to monooxygenase activity are not inducible with 3-methylcholanthrene. Tumorigenesis was not related to induction of aryl monooxygenase activity in these mice.

Epoxide hydrase activity towards styrene oxide, naphthalene oxide and benzene oxide is induced substantially in rats by pretreatment with phenobarbital and slightly by pretreatment with 3-methylcholanthrene. Attempts to inhibit epoxide hydrase activity in vivo with potent uncompetitive and noncompetitive epoxide inhibitors were unsuccessful. Such compounds do reduce glutathione levels in vivo, but this reduction had no significant effect on the arene oxide-mediated hepatotoxicity of chlorobenzene.

The metabolism of various deuterated substrates have been studied with hepatic microsomes and in vivo in rats. Phenol formation occurs in most cases with the absence of a primary isotope effect. However, formation of the meta-hydroxylation product, 3-nitrophenol, from nitrobenzene exhibits an isotope effect k_H/k_D of 1.5, indicating that an arene oxide intermediate is not involved.

An oxidative model system consisting of aqueous thiosalicyclic acid ferrous ions and oxygen has been studied further with naphthalene. In addition to naphthol, both cis- and trans-dihydrodiols and an unusual epoxide, 2,3-epoxy-4-hydroxy-1-tetralone, were isolated. Isotopic studies with O_{18} enriched water and oxygen gas provide evidence that both cis- and trans-diols are formed by the same reaction pathway, which does not involve an intermediate

epoxide or dioxetane.

The mechanism of rearrangment and the inherent stability of arene oxides are crucial an understanding of the toxicity, carcinogenicity and metabolism of such intermediates. A variety of deuterated and methylated arene oxides have been prepared and the kinetics of aromatization to phenols measured. The results provide the basis for predictions of arene oxide stability and have revealed a new mechanism for the NIH Shift which involves the intermediate formation from certain arene oxides of a 1,4-dihydroxy-1,4-dihydrobenzene derivative. Synthetic approaches to arene oxides of polycyclic aromatic hydrocarbons are under exploration.

The course of addition of various nucleophiles to benzene oxide and naphthalene oxide has been elucidated, thus providing information on the potential reactions of such arene oxides with nucleophilic moieties of macromolecular tissue constitutents.

Mono- and di-oxygenase activity has been further investigated in fungi and bacteria. Isotopic labeling with oxygen-18 established cis-dihydrodiol formation from naphthalene as typical of the action of a dioxygenase. The absolute stereochemistry of several such dihydrodiols is being investigated in terms of the fundamental basis for circular dichroism and optical rotary dispersion. Bacterial and fungal systems exhibit monooxygenase activity as demonstrated by the NIH Shift during phenol formation, the isolation of trans-dihydrodiol from naphthalene and the incorporation of oxygen-18 into phenols from molecular oxygen. Fungi contain the only known monooxygenases that appear to effect the formation of oxirane-substituted arene oxides. The fungi, Cunninghamella baineri, has a nonspecific enzyme system remarkably simple to the P-450 drug metabolizing system of liver.

Novel findings regarding the fundamental nature of through space coupling observed in nuclear magnetic resonance spectroscopy have been made using ^{13}C and ^{15}N oxaziridines, imines and nitrones. In addition, the first known cis-aldimine has been prepared.

Significance to Biomedical Research and the Program of the Institute: Enzymatic oxidation reactions are extremely important in many biosynthetic pathways and in the metabolism, detoxication and action of most drugs.

Proposed Course of Project: The mechanism(s) of enzymatic hydroxylation will be further investigated. Arene oxides of other aromatic substrates will be synthesized and attempts will be made, using tracer techniques, to demonstrate their intermediacy in aryl oxidation. The mechanism of action of epoxide hydrase and glutathione transferase and their role in drug detoxification will be further studied. An assay for "benzene oxide hydrase" will be developed and this enzyme will be investigated.

The effect of various parameters on the stereochemical opening of epoxides with the microsomal epoxide hydrases will be explored. Further studies on model systems including photolysis of N-oxides, and ferrous ion, oxygen, thiosalicylic acid will be conducted.

Honors and Awards: None

Publications:

Jerina, D. M., Kaubisch, N., and Daly, J. W.: Arene Oxides as Intermediates in the Metabolism of Aromatic Substrates: Alkyl and Oxygen Migrations during Isomerization Alkylated Arene Oxides. *Proc. Nat'l. Acad. Sci. (USA)* 68: 2545, 1971.

Oesch, F., Kaubisch, N., Jerina, D. M., and Daly, J. W.: Hepatic Epoxide Hydrase: Structure-Activity Relationships for Substrates and Inhibitors. *Biochemistry* 10: 4858, 1971.

Auret, B. J., Boyd, D. R., Robinson, P. M., Watson, C., Daly, J. W., and Jerina, D. M.: The NIH Shift during Hydroxylation of Aromatic Substrates by Fungi. *Chemical Communications*: 1585, 1971.

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ANNUAL REPORT
LABORATORY OF EXPERIMENTAL PATHOLOGY

Introduction

The research activities in our Laboratory are summarized below and, as in the past, progress in research areas are presented rather than individual activities, whenever this was possible.

The Laboratory has continued to provide tissue diagnoses on surgically obtained specimens and on selected autopsy material for the Division of Indian Health of the Public Health Service, the Hospital of the Coast Guard Academy in New London, Connecticut, and several of the Federal Prisons. As in the past, several pathologists outside our Laboratory have generously helped us in this activity. They were Dr. Ruth Kirschstein (Chief of Pathology, DBS and Asst. Director, DBS) and Dr. Francis Chisari (Laboratory of Pathology, DBS). The Laboratory gratefully acknowledges their assistance in this work.

Dr. Benjamin Highman has continued as the Liaison Officer of the Public Health Service to the Armed Forces Institute of Pathology. He is expected to return full-time to the Laboratory next year.

Biology of Degenerative Joint Diseases (Dr. Sokoloff and staff)

As in previous years, the Section on Rheumatic Diseases has continued its investigations on the biology of degenerative joint disease. This major cause of rheumatic disability involves the interaction of aging and mechanical destruction of the movable joints.

The principal effort during the past period has been directed at the behavior of articular chondrocytes in vitro and the factors that govern chondroid differentiation. Under monolayer conditions, certain phenotypic properties are preserved whereas others are altered. The chondrocytes retain a unique capacity for synthesizing sulfated mucopolysaccharides. Dr. Srivastava has found that the latter are primarily of cartilaginous type insofar as they consist primarily of chondroitin sulfate and there is little hyaluronate. This is in contrast to other connective tissue cells (skin fibrocytes) where the predominant acid mucopolysaccharide product is hyaluronate. On the other hand, Dr. Sokoloff collaborating with Drs. Layman and Miller, formerly of the NIDR, reported that collagen synthesized by the chondrocytes under monolayer conditions resembles chemically the collagen of skin and bone rather than of intact cartilage. Because articular cartilage is an avascular tissue having a predominantly glycolytic metabolism, Dr. Marcus studied the effect of hypoxic environments on the growth and radiosulfate incorporation by the chondrocytes. The latter proved more resistant to a low (7%) oxygen tension than did fibrocytes so far as growth was concerned; nevertheless, hypoxia did not prove to be a chondrogenic force as measured by chondroitin sulfate synthesis under these conditions. Cell for cell, the chondrocytes had a higher rate of glycolysis than did the fibrocytes. Rumalon, a proprietary extract of cartilage and bone marrow has been widely empolyed, particularly in Europe, in the treatment of degenerative joint disease. This form of therapy has been based

on a putative sulfation factor-like activity. No effect of rumalon was found either on DNA synthesis or radiosulfate incorporation by Mr. Malemud and Dr. Sokoloff. The chondrocyte growth factor activity, reported last year, as a contaminant of pituitary glycoprotein hormones was also found in crude human chorionic gonadotropin. This factor (or factors) has been partly characterized as a glycoprotein by Dr. Jean Hickman in collaborative studies.

The biomechanical work of the Section has been carried forward by a guest worker from Copenhagen, Dr. Inge Reimann. Using a glass-rubber bearing test system, adhesive rather than lubricating properties, were found for synovial fluid under certain conditions.

Structure and Biochemical Properties of Cell Membranes (Dr. Marchesi and staff)

Further characterization of the major glycoprotein* of the human red cell membrane. (*named Glycophorin)

During the past year progress has been made in three general areas.

Order of tryptic glycopeptides: Four unique glycopeptides are produced when the isolated glycoprotein is digested with TPCK-Trypsin. These have been purified and partially characterized in terms of amino acid and carbohydrate composition and biologic activities. Three of these peptides (labeled α-1, α-2, α-3) can be released from the membrane when intact red cells are treated with trypsin, while the fourth, called β, can only be released when the glycoprotein is extracted from the membrane. On the basis of these and other results described below we have proposed a tentative arrangement of these glycopeptides in the original glycoprotein molecule which explains some of the previously reported results obtained from digesting intact cells with proteolytic enzymes. The original studies were carried out by analysing peptide and sugar moieties by conventional assays which require relatively large amounts of material, but similar experiments have also been carried out on much smaller samples which were pre-labeled with radio isotopes (I^{125} or H^3 borohydride) with the identical results. These latter studies confirmed the earlier findings but they also demonstrated that the same approaches and techniques can be used to study membrane glycoproteins from other cell types which are considerably more difficult to obtain in large quantities. (with Jackson, Segrest, and Kahane).

Linear arrangement of the glycophorin molecule: Five unique fragments are generated when purified glycophorin is treated with cyanogen bromide. These have been purified and characterized, and on the basis of composition and overlaps with the tryptic glycopeptides, a model for their linear arrangement has been proposed in which glycophorin is depicted as a single polypeptide chain of approximately 200 amino acids. The N-terminal one-half of the polypeptide chain is rich in threonine/serine and this portion contains all of the covalently-bound carbohydrate. Some of the latter is also attached to asparagine residues. The C-terminal half of the molecule has a relatively high content of non-polar amino acids. To investigate this segment of the molecule in more detail, two peptides derived from this region were isolated and partial amino acid sequence data were obtained with an automatic sequenator. To date the

sequence of approximately one-half of the polypeptide chain has been determined as shown below.

"HYDROPHOBIC DOMAIN" OF ERYTHROCYTE GLYCOPHORIN

```
 1                5                   10                  15
VAL-GLN-LEU-ALA-   -   -PHE-SER-GLX-LEU-GLX-ILE-THR-LEU-ILE

                    T(is)
16               20                  25                  30
   -PHE-GLY-VAL-MET-ALA-GLY-VAL-ILE-GLY-THR-ILE-LEU-LEU-ILE

          T(is)

                        C-2
31               35                  40                  45
SER-TYR-GLY-ILE-ARG-ARG-LEU-LEU-LYS-LYS-SER-PRO-SER-ASP-VAL

                    C-2
46               50
LYS-PRO-LEU-PRO-SER
```

This stretch of peptide contains some 30 or more non-polar amino acids without interruption by a changed group. As far as we are aware this is a unique sequence and may well represent a hydrophobic domain of the molecule which is responsible for its tight association with membrane lipids. (with Segrest and Terry)

Orientation of glycophorin at the cell surface: In last years report freeze-etch experiments were described which indicated that the polar ends of glycophorin molecules were exposed to the cell exterior while their C-terminal segments were connected in some way with intramembranous structures. These findings have been extended by analysing the location of different segments of the polypeptide chain relative to the lipid barrier. Using the lactoperoxidase-catalysed iodination procedure as a way to label exposed peptide segments it was found that only the N-terminal one-half of the polypeptide chain is outside the lipid barrier. The C-terminal segment which includes the hydrophobic portion decribed above appears to reside partly with the lipid region of the membrane and partly within the cytoplasm of the cell. It is tentatively concluded that glycophorin molecules extend completely across the cell membrane. (with Kahane)

Histochemical and Ultrastructural Studies: Methods and Applications (Dr. Feder and staff)

Microperoxidase as an ultrastructural tracer: Ultrastructural studies have been carried out with microperoxidase (MP), a heme-peptide developed as a tracer in this laboratory. After introduction of this compound into tissues or cells, its position can be readily determined with the electron microscope. Several studies have been carried out with MP, as follows: (a) The most promising approach at present to an understanding of retinal function is the examination of individual retinal neurons in situ with electrophysiological

methods; a major technical problem has been to identify, by its morphology, any particular neuron on which electrical measurements have been made, and in particular to determine morphologically its synaptic relations with other cells. At the level of the light microscope this problem has been dealt with by means of a dye (Procion Yellow), which is injected through the recording microelectrode and observed by its fluorescence after it spreads through the cell processes. Studies are in progress to determine whether MP can be used in similar fashion to correlate the electrophysiology of the retina with its ultrastructure. The properties of MP that make this plan feasible are its low molecular weight (which permits rapid transfer of MP from microelectrode to neuron by iontophoresis and also results in its rapid spread by diffusion throughout the injected neuron), its low toxicity, and its enzyme-like activity (which permits detection of MP with the electron microscope even when it is present at low concentration) (with Dr. Schwartz and Mr. Stewart). (b) The electronic synapse in the giant axon of the crayfish is an easily studied example of the gap junction, a common type of cell-to-cell contact. This synapse is permeable to MP (mol. wt. 1900) injected intracellularly, but not to horseradish peroxidase (mol. wt. 40,000). Observations have been made with the electron microscope to determine the route by which MP moves from cell to cell across the gap junction, as well as to identify the barrier to the movement of horseradish peroxidase. Interpretation of the experiments is not simple, however, because of uncertainty over the effectiveness of glutaraldehyde fixation in the immobilization of the two tracers. This point is under study (with Dr. Reese, Dr. M.V.L. Bennett and Mr. Stewart).

Thyroid peroxidase cytochemistry: Studies are being carried out on the fine structure of the rat thyroid and on the fine structural localization of thyroid peroxidase in the thyroid follicular cells. This enzyme is demonstrated histochemically for the electron microscope by incubation of the tissue in a medium containing diaminobenzidine and a hydrogen peroxide-generating system. The fine structural changes that occur in the secretory epithelium after treatment with thyroid-stimulating hormone are accompanied by alterations in the distribution of the enzyme (Drs. Tice and Wollman).

Critique of work on scotophobin: A detailed and critical analysis has been made of a report by Ungar, Desiderio and Parr on the chemistry of scotophobin. The report and the critique will be published together in Nature (with Mr. Stewart).

Amyloidosis (Dr. Glenner and staff)

Human amyloidosis of immunoglobulin origin: Evidence obtained by amino acid sequence and immunochemical studies of purified amyloid fibril proteins suggests that amyloid fibrils consist primarily of the amino-terminal variable segment of the light polypeptide chain of homogeneous immunoglobulins in those cases studied by us (Drs. Glenner, Kimura, Isersky, Page, Harada, Zopf, Terry). If indeed this is their source, then it should be possible to produce a variable region fragment having the characteristics of amyloid fibrils from homogeneous light chains.

A method for cleaving Bence Jones proteins into variable and constant fragments by proteolysis has been described by Solomon et al. We subjected three κ and two λ Bence Jones proteins having no polysaccharide constituents from patients not known to have amyloidosis to a 2 to 5 hour incubation in glycine buffer, pH 3.5, in the presence of pepsin at 37°C. During the incubation, precipitates formed with the two λ Bence Jones proteins. These precipitates were centrifuged at 8000g for 1 hour in a refrigerated centrifuge, and a portion of the sediments was stained with alkaline Congo red, examined by polarization microscopy, and found to have a green birefringence. Electron microscopy by the negative staining method showed that the precipitates were composed of fibrils measuring from 70 to 80 Å in width and from 1000 to 2000 Å in length, and that they had the appearance of twisted doublet filaments characteristic of amyloid fibrils. The remaining sediments were thoroughly washed with 0.1N ammonium acetate buffer, pH 5.0, and lyophilized. Examination of both sediments by x-ray diffraction methods revealed a strong, sharp band at 4.75 Å and a moderately strong diffuse halo at 9.3 Å. This picture is typical of the antiparallel β-pleated sheet configuration of amyloid fibrils. Infrared studies confirmed this x-ray diffraction interpretation of the fibril conformation. One of the λ Bence Jones proteins was chosed for further study. The proteolytically derived sediment (BJ fragment) was denatured in 6M guanidine hydrochloride buffered to pH 8.0 containing mercaptoethanol, exhaustively dialyzed with distilled water, and lyophilized. The molecular weight of the BJ fragment, as determined by column chromatography on Sephadex-G-100 equilibrated with 5M guanidine in 1N acetic acid, was 4600. This protein was reduced and aminoethylated. The aminoethylated protein was digested with trypsin, and peptide mapping was performed. Common region peptides from the peptide map of the BJ fragment were absent. The amino acid sequence of the aminoethylated Nic fragment was determined with an automatic amino acid sequencer. The sequence data in conjunction with peptide mapping and molecular weight studies showed that the BJ fragment derives exclusively from a portion of the variable region of BJ protein. These studies demonstrate that fibrils having the tinctorial, ultrastructural, and crystallographic properties of amyloid fibrils can be created from some but not all Bence Jones proteins by peptic digestion at pH 3.5 and 37°C. The fact that "amyloid" fibrils can be created from Bence Jones proteins at a physiologic temperature in the presence of a proteolytic enzyme having an acidic pH optimum suggests that one possible pathogenetic mechanism for amyloid formation may be by means of intralysosomal catheptic digestion of light polypeptide chains of immunoglobulins. This mechanism is supported by the frequent close spatial relationship between amyloid deposits and cells of the macrophage system and by the electron microscopic observations of fibrils within plasmalemmal invaginations and membrane-bound vesicles of macrophage. (Drs. Glenner, Terry, Ein, Eanes, and Bladen).

Human amyloidosis of unknown origin: Further chemical analysis has shown that not all amyloid fibrils are derived from immunoglobulin light chains. Our definition of amyloid is based on certain derivative properties. It is a material deposited in tissues which exhibits Congo red polarization birefringence, has a fibrillar appearance by electron microscopy and characteristic x-ray diffraction pattern indicating a β-pleated sheet conformation. Under appropriate conditions, proteins other than light chains could assume these same

properties. An anti-parallel chain β-pleated sheet conformation has been found by infra-red studies in the Fd fragment of immunoglobulin heavy chains. Studies of amino acid polymers show that poly-l-lysine in its β-conformation has many of the properties of amyloid fibrils except for some difference in the spacing of the polypeptide chains of the β-sheet. Partial amino acid sequence determination of amyloid fibrils from one patient with classic "secondary" amyloidosis does not correspond, as of this writing, to any known protein sequence. Thus, some cases of amyloidosis may result from tissue deposition of portions of immunoglobulins other than light chains or of proteins other than immunoglobulins. (Drs. Glenner, Terry, Ein, Eanes, and Bladen).

Cytogenetic Studies (Dr. Tjio and staff)

Human cytogenetic studies: Clinical and cytogenetic studies on human congenital and developmental disorders are being pursued with investigations of cases with mosaicism, precocious puberty, failure of gonadal development and normal karyotype, gonadal deficiency secondary to hypogonadotropism, gonadal dysgenesis with mosaicism and Russel-Silver dwarfism.

Cytogenetic studies on the effect of tranquillizers and LSD on human chromosomes continues in an effort to determine if these drugs can contribute to chromosomal damage.

Cytogenetic and reproductive studies of mice with translocations and their hybrids: Normal mouse chromosomes from several strains and translocation chromosomes T1Wh, T163H, and T1A1d are being studied using the quinacrine mustard and Giemsa-banding methods. Previously, only the smallest autosome (number 19) and the Y chromosome could be identified, but with the new techniques, the specific chromosomes involved in the three translocations can be determined. Identification of specific chromosomes with known mouse linkage groups is then possible.

F_1 hybrids from crosses between T1Wh and T163H translocation homozygotes were investigated. Analysis of meiotic chromosomes of the F_1 males showed abnormal meiotic pairing (quadrivalent formation) comparable to that found in humans carrying reciprocal translocations, as well as cytological evidence of regular non-disjunction for chromosome 19 (the smallest mouse autosome). A high incidence of mice trisomic for chromosome 19 were found among the progeny of the F_1 when crossed with each other (12% at birth, 11% of fetuses), with the parent strains (7.7% to 13.8% of fetuses), and with normal non-translocation-bearing mice (17.5% of fetuses). Some trisomics had cleft palates, and all of those studied after birth were smaller than their littermates and died during the first day of life. Even without cleft palate, such mice were unable to feed; death usually resulted from respiratory distress or dehydration.

At present, trisomic fetuses are being compared to their normal littermates for the following: (1) total body weight, (2) weight of brain, lungs, and kidneys, (3) placental weight, and (4) gross and microscopic evidence of abnormal development. Of interest is the use of the trisomy 19 model to analyze the effects of autosomal imbalance on general growth and development as well as

development of specific organs. Since chromosomal imbalance in the human is often associated with infertility, microscopic studies of the gonads of newborn mice will be of special interest. Likewise, comparison of the gross and microscopic characteristics of trisomic and normal fetal mouse brain may provide a model for understanding the basis of the profound effects of autosomal imbalance on human brain development and function. To date, our weight data indicate that, in trisomy 19, growth retardation begins soon after implantation and that the birth and other specific organs are uniformly reduced in size and weight. Microscopic and placental weight studies are not completed. To our knowledge, this system is the first example described in mammals in which trisomy for a specific autosome can be regularly produced. In addition to its value as a model to study how chromosomal imbalance produces anomalies in the human, it also is a system where the incidence of nondisjunction has been directly evaluated (cytologically in the F_1 hybrid, and in specific crosses) and where the effects of such factors as age, parity, and sex on nondisjunction can be tested.

F_1 hybrids from crosses between mice homozygous for T1Wh and T1A1d have also been studied. These hybrids also have an increased incidence of nondisjunction, but only one trisomic (chromosome 19) has been detected among their progeny at birth. Studies initiated last year to derive a strain of mice homozygous for both T1Wh and T1A1d with a reduced chromosome number of 36 (normal number = 40) from the F_1 hybrids have been completed. To date, the new strain has been fertile and has shown no evidence of nondisjunction; average size of the first 10 litters was 6.1. The accumulation of translocations of the centric fusion (Robertsonian) type, with reduction of chromosome number by one for every added translocation chromosome (and maintenance of a constant number of chromosome arms), has long been considered as a possible evolutionary mechanism. Presumably, this rearrangement in chromosomal material would be accompanied by progressive changes in the animal. Starting with a mixed colony of mice with normal chromosome number (40) and chromosome number of 39, the T1Wh strain with chromosome number of 38 was established in 1967 after crossing only the animals with 39 chromosomes. This past year, the addition of 2 more translocation chromosomes (T1A1d/T1A1d) has resulted in a still lower chromosome number (36) without any obvious deleterious effects. Further studies to compare the parent strains 38, (TW/TW and TA/TA) with the new strain (36) (TW/TW TA/TA) need to be done in order to test the significance of our breeding experiments with regard to specific evolutionary theory. However, this new system to produce such changes in the laboratory provides an opportunity to directly study the problem.

Environmental Cross Adaptation Study (Dr. Highman)

In a study with Dr. Altland on cross adaptation between cold acclimation and hypoxia, groups of 2 cold-acclimated and 2 unacclimated rats were exposed simultaneously for 4 hours at room temperature in a plastic chamber to O_2 - N_2 concentrations of 8.3, 6.6, and 5.4% O_2 equivalent to 23,000, 28,000 and 32,000 feet altitude, respectively. No significant differences were noted between the 2 groups at 8.3% O_2, but at 6.6% O_2, the cold-acclimated group showed re-

duced tolerance to hypoxia. Elevations in serum levels of glutamic oxalacetic and pyruvic transaminases, aldolase, lactic dehydrogenase, and creatine phosphokinase were up to 6 times greater in the cold-acclimated than in the unacclimated rats immediately after the exposure to 6.6% O_2. The acclimated rats also developed a significantly greater increase in the incidence of fatty changes in the striated muscles and of marked depletion of hepatic glycogen. Centrilobular necrosis of liver cells and focal necrosis of renal tubules were seen in 58 and 29% of the cold-acclimated and in only 21 and 0% of the unacclimated rats, respectively. During an exposure of 5.4% O_2, the mortality rate was 100% in the acclimated and only 30% in the unacclimated rats. The lesser tolerance of the cold-acclimated rats to hypoxia is attributed to the persistence of an increased metabolic rate induced by cold. These findings are of importance because of the frequency of such dual human exposure as, for example, in the Chinese-Indian-Himalayan confrontation a few years ago.

Studies on Bacterial Endocarditis (Dr. Highman)

It was shown previously that epinephrine greatly increases the susceptibility of x-irradiated rats to bacterial endocarditis. To determine if this was due to the effect of a nonspecific dual stress or was specifically related to circulatory insufficiency caused by the myocarditis induced by epinephrine, 3 of 4 groups of rats received 2 whole body exposures to 400 R in 4 days, and, 3 and 4 days later, all rats received an i.v. inoculation of Streptococcus mitis. The nonirradiated and one irradiated group received 40 mg/kg of isoproterenol subcutaneously 24 hours before and immediately after the first bacterial inoculation to produce myocardial lesions. A second irradiated group received 1.5 ml/kg of 50% CCl_4 in olive oil by stomach tube 6 hours before the first bacterial inoculation to produce necrosis of the liver and a dual stress without myocardial lesions. The third group served as an irradiated control. The mean leukocyte count dropped from about 4000/mm^3 just before inoculation to 1700/mm^3 6 days later. At 6 days after inoculation, the nonirradiated group had no bacterial lesions while the incidence of bacterial endocarditis and mural vegetations was significantly increased in the irradiated rats given isoproterenol, but not in the CCl_4 group, even though this group had a higher morbidity and mortality rate. This indicates that the increased susceptibility to endocarditis was not due to a nonspecific dual stress, but was specifically related to the myocardial lesions induced by the isoproterenol. Previous investigators have attributed these lesions to hypoxia, resulting from the cardiostimulative effect of isoproterenol, which greatly increases the myocardial oxygen requirements, and to peripheral vasodilatation, which reduces the systemic blood pressure and intramyocardial capillary pressure and blood flow below requirements for adequate tissue perfusion. It is postulated that the reduced cardiac microcirculation and tissue perfusion, in combination with severe radiation leukopenia, prevents sufficient leukocytes and other defensive humoral factors from reaching the valvular leaflets and the endocardium in time to prevent or overcome infection. The findings also suggest that myocardial circulatory insufficiency, secondary to congenital and acquired valvular deformities, may be an important factor in a combination of factors predisposing to bacterial endocarditis in man.

Cyasin Research (Dr. Laqueur)

Several years ago, at an early stage of research in cycasin toxicity and carcinogenicity, it was reported that crude cycad material when fed to pregnant rats would induce tumors in the offspring. Subsequent studies showed that cycasin, the toxic glycoside in crude cycad materials, and the aglycone of cycasin, methylazoxymethanol (MAM), had crossed the placenta and could be detected in fetal tissues. On the basis of these observations and the additional finding that the compound and the nucleic acids of fetal tissues had reacted, a direct effect of the compound on fetal tissues and their components could be assumed.

The original experiment in which crude cycad meal was fed, did permit only a rough estimation of the amount of cycasin which the rats had ingested. After establishing MAM as the proximate carcinogen in a series of experiments using germ-free rats, it was decided to reinvestigate the problem of transplacental carcinogenesis with MAM and to compare these results with those obtainable with chemically related compounds such as methyl-and ethylnitrosourea and elaiomycin.

During the past year microscopic examination of tissues of rats was begun which had been obtained from animals exposed to MAM at various times during fetal development. The results which ultimately will be based on detailed examination of several hundreds of animals, is in progress and should be completed next year. Only a few observations can be cited now which are not likely to change drastically when the entire study is completed. (1) Intraperitoneal injection of pregnant rats with MAM shortly before parturition induces pulmonary tumors in a high percentage of the progeny. This same effect was previously noted in newborn rats exposed to the carcinogen but only infrequently in older animals. Apparently, the perinatal period is particularly suitable for induction of pulmonary neoplasms with this compound. (2) Induction of central nervous system tumors is possible over a considerably greater span and several such tumors have occurred in animals exposed to MAM as early as the beginning of the second week of intrauterine development. (3) An appreciable number of proliferative and neoplastic diseases of the lymphoreticular system has been observed in old rats whether exposed to the carcinogen or serving as controls. The development of this condition appears to be related to age but has not been described in aged rats of the Fischer strain, which was exclusively used in this series of experiments. (4) Elaiomycin has been found to transplacentally induce tumors including those of the central nervous system.

Serial No. NIAMD-LEP-1
1. Experimental Pathology
2. Office of the Chief
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Metabolic and carcinogenic effects of Cycas Circinalis, its glucoside cycasin and its aglycone, methylazoxymethanol, in conventional and germfree rats.

Previous Serial Number: Same

Principal Investigator: Gert L. Laqueur

Other Investigators: Dr. M. Spatz, Dr. H. Matsumoto, and Mr. E. G. McDaniel

Cooperating Units: LNNS-NINDS (Dr. Spatz)
LNE-NIAMD (Mr. McDaniel)
University of Hawaii (Dr. Matsumoto)

Man Years:

Total: 4
Professional: 1
Other: 3

Project Description:

The study dealing with the growth-inhibiting effect of methylazoxymethanol (MAM) on the fetal brain in rats was completed and prepared for publication. It was shown that DNA synthesis was reduced during the first 3 days after injection of the compound into the mother rat. The MAM treated brain grew at almost the normal rate after this period, but the reduction in DNA persisted through maturity of the animal. The difference in DNA content between normal and microencephalic brain was restricted to the cerebral hemispheres. In particular, it did not affect the postnatal growth of the cerebellar cortex (Matsumoto, Spatz and Laqueur).

Following preveious studies on transplacental carcinogenesis after feeding crude cycad preparations to pregnant Sprague-Dawley rats (Spatz and Laqueur, 1967), a new study was under taken to examine this effect with MAM in Fischer rats and to compare the results with those which might be obtained with methylnitrosourea, ethylnitrosourea and elaiomycin. The study which is still incomplete has however, produced several new findings which are not likely to change after completion. (1) Elaiomycin has induced tumors by the transplacental route. (2) Pulmonary tumors are most readily induced with MAM during late fetal life, i.e. within 24-48 hrs before birth but

rarely earlier. (3) Induction of tumors of the central nervous system with MAM is much less restricted in time and can be observed in rats exposed to the compound during the second and third weeks of pregnancy. (4) Hyperplastic and neoplastic changes in bone marrow and lymphoreticular system have been observed in old Fischer rats of both sexes irrespective of whether they were treated or served as controls. The microscopic work-up of this material which consists of several hundreds of animals should be completed during the next year.

Publication:

Matsumoto, H., Spatz, M. and Laqueur, G. L.: Quantitative changes with age in the DNA content of methylazoxymethanol-induced microencephalic rat brain. J. Neurochemistry 19: 297-306, 1972.

Serial No. NIAMD-LEP-2a
1. Experimental Pathology
2. Office of the Chief
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Histopathologic, serum enzyme and other changes produced in animals by various environmental and other stresses.

Previous Serial Number: Same

Principal Investigator: Benjamin Highman

Other Investigators: Drs. P. D. Altland, M. P. Dieter, H. M. Maling

Cooperating Units: LPB-NIAMD (Altland, Dieter - LBO-20)
LCP-NHLI (Maling)

Man Years:

Total: 1.5
Professional: .5
Other: 1

Project Description:

Environmental Cross Adaptation Study: In a study with Dr. Altland on cross adaptation between cold acclimation and hypoxia, groups of 2 cold-acclimated and 2 unacclimated rats were exposed simultaneously for 4 hours at room temperature in a plastic chamber to O_2-N_2 concentrations of 8.3, 6.6, and 5.4% O_2 equivalent to 23,000 28,000 and 32,000 feet altitude, respectively. No significant differences were noted between the 2 groups at 8.3% O_2, but at 6.6% O_2, the cold-acclimated group showed reduced tolerance to hypoxia. Elevations in serum levels of glutamic oxalacetic and pyruvic transaminases, aldolase, lactic dehydrogenase, and creatine phosphokinase were up to 6 times greater in the cold-acclimated than in the unacclimated rats immediately after the exposure to 6.6% O_2. The acclimated rats also developed a significantly greater increase in the incidence of fatty changes in the striated muscles and of marked depletion of hepatic glycogen. Centrolobular necrosis of liver cells and focal necrosis of renal tubules were seen in 58 and 29% of the cold-acclimated and in only 21 and 0% of the unacclimated rats, respectively. During an exposure to 5.4% O_2, the mortality rate was 100% in the acclimated and only 30% in the unacclimated rats. The lesser tolerance of the cold-acclimated rats to hypoxia is attributed to the persistence of an increased metabolic rate induced by cold. These findings are of importance because of the frequency of such dual human exposure as, for example, in the Chinese-Indian-Himalayan confrontation a few years ago. Additional cooperative studies in progress on cross adaptation are detailed elsewhere by Dr. P. D. Altland (LPB-NIAMD).

Hepatotoxic Studies: It is well known that hepatic necrosis developing after carbon tetrachloride (CCl_4) administration is preceded by an accumulation of fat in the liver and a fall in plasma triglycerides, but it is not known whether similar changes precede the development of necrosis after administration of other hepatotoxic agents such as allyl alcohol, bromobenzene and thioacetamide. The purpose of this study was to explore the possible correlations between a fall in plasma and a rise in liver triglyceride levels (fatty changes) and the development of hepatic necrosis, and to determine if potentiation or inhibition of triglyceride accumulation in the liver by pretreatment with phenobarbital or various blocking compounds would potentiate or inhibit necrosis. One of the preliminary findings was that pretreatment with Dibenamine, an alpha adrenergic blocking agent, reduced markedly the hepatotoxicity of CCl_4 in rats, as evident from decreased liver fatty changes and necrosis, smaller elevations in plasma glutamic-pyruvic transaminase, (GPT), and prevention of the early fall in plasma triglycerides. Dibenamine pretreatment also reduced the elevations in plasma GPT and liver triglycerides induced by thioacetamide, but did not affect the toxicity of allyl alcohol. There was evidence that Dibenamine inhibited the binding of CCL_4 to liver phospholipids and that this, rather than its blocking of alpha adrenergic receptors, probably contributed to its protective action. Further details concerning this project are given elsewhere by Dr. H. M. Maling (LCP-NHLI).

Honors and Awards:

Delegate to the House of Delegates of the College of American Pathologists for the PHS.

Member of the Executive Committee and Vice Chairman of the Section on Governmental Pathology, College of American Pathologists.

Publications:

Altland, P, D., and Highman, B.: Effects of polycythemia and altitude hypoxia on rat heart and exercise tolerance. Am. J. Physiol. 221: 388-393, 1971.

Altland, P. D., Highman, B., and Dieter, M. P.: Reduced hypoxic tolerance of cold-acclimated rats: Serum enzyme and tissue changes. Am. J. Physiol. (in press).

Serial No. NIAMD-LEP-2b
1. Armed Forces Institute of Pathology
2. Radiopathology Division
3. Washington

PHS-NIH-AFIP
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title:
1. Experimental bacterial endocarditis following x-irradiation.
2. Serum enzyme and pathologic changes after whole body irradiation and effect of adrenergic, hepatotoxic and blocking agents.
3. Characterization of the anatomic, histologic, pathologic and selected physiologic attributes of Mystromys.
4. Radiological-pathological correlations - Changes induced by thorotrast.

Previous Serial Number: Same

Principal Investigator: Benjamin Highman

Other Investigators: CPT N. T. Byers, CPT W. H. Cyr, CPT R. P. Streett, Jr., and Drs. M. Rodrigues and B. S. Fine

Cooperating Units: Radiation Pathology Branch, AFIP (Byres, Cyr, and Streett)
Ophthalmic Pathology Branch AFIP (Rodrigues, Fine)

Man Years:

Total: 1.5
Professional: .5
Other: 1

Project Descriptions:

Bacterial Endocarditis After X-irradiation: It was shown previously that epinephrine greatly increases the susceptibility of x-irradiated rats to bacterial endocarditis. To determine if this was due to the effect of a nonspecific dual stress or was specifically related to circulatory insufficiency caused by the myocarditis induced by epinephrine, 3 of 4 groups of rats received 2 whole body exposures to 400 R in 4 days, and 3 and 4 days later, all rats received an i.v. inoculation of Streptococcus mitis. The nonirradiated and one irradiated group received 40 mg/kg of isoproterenol subcutaneous 24 hours before and immediately after the first bacterial inoculation to produce myocardial lesions. A second irradiated group received 1.5 ml/kg of 50% CCl_4 in olive oil by stomach tube 6 hours before the first bacterial inoculation to produce necrosis of the liver and a dual stress without myocardial lesions. The third group served as an irradiated control. The mean leukocyte count dropped from about 4000/mm^3 just before inoculation to 1700/mm^3 6 days later. At 6 days after inoculation, the

nonirradiated group had no bacterial lesions, while the incidence of bacterial endocarditis and mural vegetations was significantly increased in the irradiated rats given isoproterenol, but not in the CCl_4 group, even though this group had a higher morbidity and mortality rate. This indicates that the increased susceptibility to endocarditis was not due to a nonspecific dual stress, but was specifically related to the myocardial lesions induced by the isoproterenol. Previous investigators have attributed these lesions to hypoxia, resulting from the cardiostimulative effect of isoproterenol, which greatly increases the myocardial oxygen requirements, and to peripheral vasodilatation, which reduces the systemic blood pressure and intramyocardial capillary pressure and blood flow below requirements for adequate tissue perfusion. It is postulated that the reduced cardiac microcirculation and tissue perfusion, in combination with severe radiation leukopenia, prevents sufficient leukocytes and other defensive humoral factors from reaching the valvular leaflets and the endocardium in time to prevent or overcome infection. The findings also suggest that myocardial circulatory insufficiency, secondary to congenital and acquired valvular deformities, may be an important factor in a combination of factors predisposing to bacterial endocarditis in man. Further studies are in progress to determine the effect of smaller doses of isoproterenol and pretreatment with adrenergic blocking agents and the effect of alpha adrenergic agents and other compounds on the susceptibility of irradiated rats to endocarditis.

Enzyme Changes: Studies are nearing completion on the effect of x-irradiation in rats on the toxicity of carbon tetrachloride (CCl_4) and certain other hepatotoxic agents and drugs. In general, it was found that x-irradiation greatly augments the serum enzyme elevations and the pathologic changes induced by CCl_4. This has a bearing on the use of drugs affecting the liver in radiotherapy cases or in individuals otherwise exposed to x-irradiation.

Studies on Mystromys albicaudatus (the African White-Tailed Rat): In a collaborative study with the Ophthalmic Pathology Branch of the AFIP, a light and electron microscopic study was completed on a selected group of Mystromys albicaudatus manifesting partial oculocutaneous albinism. Two papers on this subject have been published. Studies are continuing on the appearance of tumors and other lesions in a group of aged Mystromys and on a group of irradiated Mystromys. Several tumors have already been noted in both groups. A study on the effects of a high cholesterol diet fed to Mystromys is nearing completion.

Radiological-pathological Correlations - Changes Induced by Thorotrast: A study set comprising a syllabus and 90 lantern slides was prepared from material in the AFIP files to aid pathologists and radiologists in recognizing lesions induced by thorotrast. Thorotrast is a 25% colloidal suspension of thorium dioxide which was first introduced in 1928 by German radiologists for gastrointestinal x-ray studies. Because of its high x-ray density, apparent chemical inertness, and rapid uptake by the reticuloendothelial system, it soon became widely used, particularly in arteriography and hepatosplenography and in outlining sinus tracts. ^{232}Thorium is prim-

arily an alpha emitter with an estimated biological half-life of 400 years. American nuclear scientists recognized its biological hazards, but this was not fully communicated to radiologists until the late 40's when reports of liver tumors and other serious delayed effects of thorotrast began to appear in the literature. Because the latent period for such delayed effects may exceed 30 years and because as many as 100,000 people, often unknowingly, may have received thorotrast before its use was largely discontinued in 1950, it is likely that many more thorotrast lesions will appear in the future and will be seen by radiologists and pathologists who may not have encountered such lesions in the past. We have included in this set copies of some x-ray films as well as gross and microscopic pictures and have attempted to correlate the radiologic with the pathologic findings. The set includes early as well as late changes and illustrates lesions in various organs such as the liver, spleen, bone marrow and lymph nodes. The histopathologic features of thorotrast-induced hemangioendothelial sarcomas, cholangiocarcinomas and hepatocellular carcinomas are depicted as well as of thorotrastomas resulting from extravasation of injected thorotrast into the soft tissues. This set is of importance because many patients do not know that they had received thorotrast, and early diagnosis by an alerted radiologist or pathologist examining an x-ray film or biopsy may be helpful to the patient and in furthering our knowledge on the long term effects of internal alpha emitters.

Publications:

Streett, R. P., Jr. and Highman, B.: Blood chemistry values in normal Mystromys albicaudatus and Osborne-Mendel rats. Lab Animal Sci. 21: 394-398, 1971.

Rodrigues, M., Streett, R. P., Jr., and Highman, B.: Partial ocular albinism in Mystromys albicaudatus (African White-Tailed Rat). Arch. Path. 92: 212-218, 1971.

Rodrigues, M., Fine, B. S., Highman, B., and Streett, R. P., Jr.: Partial ocular albinism in Mystromys albicaudatus. (The African White-Tailed Rat) An electron microscopic study. Arch Opthal. 87: 337-346, 1972.

Highman, B., Streett, R. P., Jr., and Byers, N. T.: Effect of isoproterenol on susceptibility of x-irradiated rats to experimental bacterial endocarditis. Research Communications in Chemical Pathology and Pharmacology. (in press).

Highman, B.: Pathologic changes induced by thorotrast. A study set with syllabus and 90 lantern slides. American Registry of Pathology. (in press).

Serial No. NIAMD-LEP-3
1. Experimental Pathology
2. Office of the Chief
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Preparation of stained tissue sections for investigation and diagnostic purposes.

Previous Serial Number: Same

Principal Investigator: Mr. Walter Rawlings - Head, Tissue Preparation Laboratory

Other Investigators: None

Man Years:

Total: 4
Professional: 0
Other: 4

Project Description:

The statistical report of this unit is shown below.

	Specimen Accessioned	Stained Slides Routine	Stained Slides Special	Spare Slides	Total
Animals	1342	8700			
Surgical	1558	2660			
Autopsy	1	4			
		11364	5747		
		17111		18503	35614

Serial No. NIAMD-LEP-4
1. Experimental Pathology
2. Chemical Pathology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Isolation and study of membrane proteins.

Previous Serial Number: Same

Principal Investigator: Vincent T. Marchesi

Other Investigators: Drs. Segrest, Hourani, Kahane and Tomita

Cooperating Unit: NCI-I (Dr. Terry)

Man Years:

Total: 6
Professional: 3
Other: 3

Project Description:

Further characterization of the major glycoprotein* of the human red cell membrane. (*named glycophorin): During the past year progress has been made in three general areas. 1.) Order of tryptic glycopeptides. Four unique glycopeptides are produced when the isolated glycoprotein is digested with TPCK-Trypsin. These have been purified and partially characterized in terms of amino acid and carbohydrate composition and biologic activities. Three of these peptides (labeled α-1, α-2, α-3) can be released from the membrane when intact red cells are treated with trypsin, while the fourth, called β, can only be released when the glycoprotein is extracted from the membrane. On the basis of these and other results described below we have proposed a tentative arrangement of these glycopeptides in the original glycoprotein molecule which explains some of the previously reported results obtained from digesting intact cells with proteolytic enxymes. The original studies were carried out by analysing peptide and sugar moieties by conventional assays which require relatively large amounts of material, but similar experiments have also been carried out on much smaller samples which were pre-labeled with radio isotopes (I^{125} or H^3 borohydride) with the identical results. These latter studies confirmed the earlier findings but they also demonstrated that the same approaches and techniques can be used to study membrane glycoproteins from other cell types which are considerably more difficult to obtain in large quantities. (with Jackson, Segrest and Kahane).

2.) Linear arrangement of the glycophorin molecule. Five unique fragments are generated when purified glycophorin is treated with cyanogen bromide. These have been purified and characterized, and on the basis of composition and overlaps with the tryptic glycopeptides, a model for their linear arrangement has been proposed in which glycophorin is depicted as a single polypeptide chain of approximately 200 amino acids. The N-terminal one-half of the polypeptide chain is rich in threonine/serine and this portion contains all of the covalently-bound carbohydrate. Some of the latter is also attached to asparagine residues. The C-terminal half of the molecule has a relatively high content of non-polar amino acids. To investigate this segment of the molecule in more detail two peptides derived from this region were isolated and partial amino acid sequence data were obtained with an automatic sequenator. To date we have determined the sequence of approximately one-half of the polypeptide chain as shown below.

"Hydrophobic Domain" of Erythrocyte Glycophorin

```
 1                5                        10                       15
VAL-GLN-LEU-ALA-   -   -PHE-SER-GLX-LEU-GLX-ILE-THR-LEU-ILE

————————————————————— T(is) ———————————————————————————

16               20                       25                       30
   -PHE-GLY-VAL-MET-ALA-GLY-VAL-ILE-GLY-THR-ILE-LEU-LEU-ILE

————————————————————— T(is) ———————————————————

                  ——————————————— C-2———————————————
31               35                       40                       45
SER-TYR-GLY-ILE-ARG-ARG-LEU-LEU-LYS-LYS-SER-PRO-SER-ASP-VAL

————————————————————————— C-2———————————————————————

46               50
LYS-PRO-LEU-PRO-SER
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This stretch of peptide contains some 30 or more non-polar amino acids without interruption by a changed group. As far as we are aware this is a unique sequence and may well represent a hydrophobic domain of the molecule which is responsible for its tight association with membrane lipids. (with Segrest and Terry)

3.) Orientation of glycophorin at the cell surface. In last years report freeze-etch experiments were described which indicated that the polar ends of glycophorin molecules were exposed to the cell exterior while their C-terminal segments were connected in some way with intramembranous structures. These findings have been extended by analysing the location of different segments of the polypeptide chain relative to the lipid barrier. Using the lactoperoxidase-catalysed iodination procedure as a way to label exposed peptide segments it was found that only the N-terminal one-half of the polypeptide chain is outside the lipid barrier. The C-terminal segment

which includes the hydrophobic portion described above appears to reside partly with the lipid region of the membrane and partly within the cytoplasm of the cell. It is tentatively concluded that glycophorin molecules extend completely across the cell membrane. (with Kahane)

Publications:

Marchesi, V. T.: Some properties of membrane glycoproteins. Monograph published in Cell Membranes: Biological and Pathological Aspects by The American Association of Pathologists and Bacteriologists. 1971, pp. 145-150

Nicolson, G. L., Marchesi, V. T., and Singer, S. J.: The localization of spectrin on the inner surface of human red blood cell membranes by ferritin-conjugated antibodies. J. Cell Biol. 51: 265-272, 1971.

Segrest, J. P., Jackson, R. L., Andrews, E. P., and Marchesi, V. T.: Human erythrocyte membrane glycoprotein: A re-evaluation of the molecular weight as determined by SDS polyacrylamide gel electrophoresis. Biochem. Biophys. Res. Comm. 44: 390-395, 1971.

Scott, R. E., and Marchesi, V. T.: Structural changes in membranes of transformed lymphocytes demonstrated by freeze-etching. Cell. Immuno. 3: 301-317, 1972.

Marchesi, V. T., and Andrews, E. P.: Glycoproteins: Isolation from cell membranes with lithium diiodosalicylate. Science 174: 1247-1248, 1971.

Marchesi, V. T., Tillack, T. W., Jackson, R. L., Segrest, J. P., and Scott, R. E.: Chemical characterization and surface orientation of the major glycoprotein of the human erythrocyte membrane. Proc. Nat. Acad Sci., (in press)

Tillack, T. W., Scott, R. E., and Marchesi, V. T.: The structure of erythrocyte membranes studied by freeze-etching II. Localization of receptors for phytohemagglutinin and influenza virus to the intramembranous particles. J. Exp. Med., (in press)

Marchesi, V. T., Segrest, J. P., and Kahane, I.: Molecular features of human erythrocyte glycophorin. Monograph in International Chemical and Nuclear Corp. Symposium on Biological Membrane, 1972 (in press)

Marchesi, V. T.: Isolation of membrane-bound glycoproteins with lithium diiodosalicylate. in Ginsberg, V. (Ed.): Methods in Enzymology,Vol. 28, 1972 (in press)

Marchesi, V. T.: Purification of wheat germ agglutinin by affinity chromatography. in Ginsberg, V. (Ed.): Methods in Enzymology, Vol. 28, 1972, (in press)

Serial No. NIAMD-LEP-5
1. Experimental Pathology
2. Chemical Pathology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Molecular Anatomy of Human Erythrocyte Glycophorin

Previous Serial Number: None

Principal Investigator: Jere P. Segrest

Other Investigators: Drs. Vincent T. Marchesi and Itzhak Kahane

Cooperating Units: None

Man Years:

Total: 1
Professional: 1
Others: 0

Project Description:

For the past three years this laboratory has been concerned with the structural and functional properties of cell membrane glycoproteins. The major glycoprotein of the red cell membrane, called glycophorin, has been isolated, purified and its general "molecular topography" characterized by analysis of chemical (CNBr) and enzymatic (trypsin) fragments. Glycophorin has a molecular weight of approximately 50,000, a polypeptide chain length of 206 residues and is 60% carbohydrate by weight (~ 125 residues); a number of antigenic and receptor activities are associated with these carbohydrate side chains.

Glycophorin can be divided into three regions or "domains" of distinct chemical compositions, a receptor or carbohydrate containing N-terminal half exposed to the external aqueous environment of the cell, a middle region ("hydrophobic domain") of 34 non-polar amino acid residues, and a C-terminal third which is hydrophilic and rich in proline but which contains no detectable carbohydrate. On the basis of labeling experiments utilizing lactoperoxidase catalyzed iodinization, the C-terminal region of glycophorin appears to be exposed to the cytoplasm of the red cell suggesting that the "hydrophobic domain" spans the lipophilic center of the membrane.

We are in the process of sequencing portions of erythrocyte glycophorin. Sequences obtained to date include approximately 20-30 residues at the N-terminal end of the molecule (which indicates selective heterogeneity of certain residues), 50 residues in the middle of the molecule including

a totally non-polar stretch of 34 residues ("hydrophobic domain"), and approximately 20 residues of a proline-rich, tryptic peptide believed to be C-terminal.

Work is currently in progress to investigate several working hypotheses suggested by our proposed model for the molecular anatomy of glycophorin. These possibilities which correlate structure with function include: (a) a transmembrane link suitable for the transmission of messages across the lipid barrier (b) specific binding sites for lipids (c) a helical structure for the "hydrophobic domain" similar to the transmembrane ion channel suggested for gramicidin A by Urry.

Publications:

Segrest, J. P., Jackson, R. L., Andrews, E. P., and Marchesi, V. T.: Human erythrocyte membrane glycoprotein: A re-evaluation of the molecular weight as determined by polyacrylamide gel electrophoresis. Biochem. Biophys. Res. Comm., 44: 390-395, 1971.

Segrest, J. P., and Jackson, R. L.: Molecular weight determinations of glycoproteins by polyacrylamide gel electrophoresis in sodium dodocyl-sulfate. In Ginsberg, V. (Ed.): Methods in Enzymology, Vol. 28 (in press)

Marchesi, V. T., Tillack, T. W., Jackson, R. L., Segrest, J. P., and Scott, R. E.: Chemical characterization and orientation of the major glycoprotei of the human red cell membrane. Proc. Nat. Acad. Sci. (in press).

Marchesi, V. T., Segrest, J. P., and Kahane, I.: Molecular features of human erythrocyte glycophorin. Proceedings of the California Membrane Conference (1972). (in press).

Serial No. NIAMD-LEP-6
1. Experimental Pathology
2. Biophysical Histology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Histochemistry: principles, methods and applications.

Previous Serial Number: NIAMD-LEP-8

Principal Investigator: Ned Feder

Other Investigators: Michael V. L. Bennett, Thomas S. Reese, Eric A. Schwartz, Walter W. Stewart.

Cooperating Units: LNS-NINDS (Reese)
LN-NINDS (Schwartz)
Albert Einstein Medical School (Bennett)

Man Years:

Total: 5
Professional: 2
Other: 3

Project Description:

Microperoxidase as an ultrastructural tracer.

Ultrastructural studies have been carried out with microperoxidase (MP), a heme-peptide developed as a tracer in this laboratory. After introduction of this compound into tissues or cells, its position can be readily determined with the electron microscope. Several studies have been carried out with MP, as follows.

Marking neurons for electron microscopy.

The most promising approach at present to an understanding of retinal function is the examination of individual retinal neurons in situ with electrophysiological methods; a major technical problem has been to identify, by its morphology, any particular neuron on which electrical measurements have been made, and in particular to determine morphologically its synaptic relations with other cells. At the level of the light microscope this problem has been dealt with by means of a dye (Procion Yellow), which is injected through the recording microelectrode and observed by its fluorescence after it spreads through the cell processes. Studies are in progress to determine whether MP can be used in similar fashion to correlate the electrophysiology of the retina

with its ultrastructure. The properties of MP that make this plan feasible are its low molecular weight (which permits rapid transfer of MP from microelectrode to neuron by iontophoresis and also results in its rapid spread by diffusion throughout the injected neuron), its low toxicity, and its enzyme-like activity (which permits detection of MP with the electron microscope even when it is present at low concentration) (with Dr. Schwartz and Mr. Stewart).

Substructure of an electrotonic synapse.

The electrotonic synapse in the giant axon of the crayfish is an easily studied example of the gap junction, a common type of cell-to-cell contact. This synapse is permeable to MP (mol. wt. 1900) injected intracellularly, but not to horseradish peroxidase (mol. wt. 40,000). Observations have been made with the electron microscope to determine the route by which MP moves from cell to cell across the gap junction, as well as to identify the barrier to the movement of horseradish peroxidase. Interpretation of the experiments is not simple, however, because of uncertainty over the effectiveness of glutaraldehyde fixation in the immobilization of the two tracers. This point is under study (with Dr. Reese, Dr. M.V.L. Bennett and Mr. Stewart).

Critique of work on scotophobin.

A detailed and critical analysis has been made of a report by Ungar, Desiderio and Parr on the chemistry of scotophobin. The report and the critique will be published together in Nature (with Mr. Stewart).

Publications:

Feder, N.: Microperoxidase: An ultrastructural tracer of low molecular weight. J. Cell Biol. 51: 339-343, 1971.

Stewart, W.W.: Comments on Isolation, identification and synthesis of a specific-behavior-inducing brain peptide by Drs. Ungar, Desiderio and Parr. Nature, (in press).

Serial No. NIAMD-LEP-7
1. Experimental Pathology
2. Biophysical Histology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Thyroid peroxidase cytochemistry.

Previous Serial Number: NIAMD-LEP-10

Principal Investigator: Lois W. Tice

Other Investigators: Dr. Seymour Wollman

Cooperating Units: LP-NCI

Man Years:

Total: 1
Professional: 1
Other: 0

Project Description:

Factors affecting the activity and localization of thyroid peroxidase, the enzyme responsible for iodination of thyroglobulin, were investigated using cytochemical methods. Thyroid epithelial cells contain an active monoamine oxidase, which has been suggested to be the H_2O_2 source for thyroperoxidase *in vivo*. This enzyme appears, in the light microscope, to have a mitochondrial distribution. Although endogenous peroxidase activity in thyroid epithelial cells (activity in the absence of added H_2O_2) considered to reflect H_2O_2 production, was inhibited by MAO inhibitors reported not to affect purified thyroperoxidase *in vitro*, attempts to augment peroxidase activity in thyroid epithelial cells with MAO substrates and/or activators have not been successful to date.

The effects of TSH on the distribution of thyroperoxidase were investigated, with particular reference to pseudopods and intracellular colloid droplets. Since peroxidase activity at the apical cell surface is at the lower limit of sensitivity of cytochemical peroxidase methods, the effectiveness of various procedures designed to increase the sensitivity of cytochemical peroxidase methods have been evaluated.

Publications:

Tice, L., and Wollman, S.: Ultrastructural localization of peroxidase activity on some membranes of the typical thyroid epithelial cell. Lab Invest. 26: 63-73, 1972.

Serial No. NIAMD-LEP-8
1. Experimental Pathology
2. Rheumatic Diseases
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Pathogenesis of experimental arthritis and pathology of rheumatism.

Previous Serial Number: NIAMD-LEP-11

Principal Investigator: Leon Sokoloff, M.D.

Other Investigators: Ralph E. Marcus, M.D.
Vishwa M.L. Srivastava, Ph.D.

Cooperating Unit: NIAMD:LBM (Jean W. Hickman, Ph.D.)

Man Years:

Total: 5 5/6
Professional: 2 5/6
Other: 3

Project Description:

Objectives:

1) Investigation of factors influencing development of degenerative joint disease in experimental animals and man.

2) Extending descriptive pathology of human rheumatic disease.

Methods Employed:

1) Articular chondrocytes are cultured in vitro and synthesis of specific cell products is measured.

2) Rheological and lubricating properties of synovial fluid are compared using specially designed apparatus.

Major Findings:

1) Some phenotypic characteristics of articular chondrocytes are preserved in monolayer culture. These cells have a unique capacity for making sulfated mucopolysaccharides. The predominant mucopolysaccharide synthesized is chondroitin sulfate, and there is little hyaluronate. In skin fibroblast cultures the reverse is true. Chondrocytes that lacked a stainable extracellular matrix in monolayer, displayed large deposits of stainable metachromatic mucopolysaccharide when transferred to spinner conditions.

2) Chondrocytes synthesized collagen provided that ascorbic acid (50μg/ml) was present in the medium. The collagen formed in vitro by whole articular cartilage was composed of α1 chains only, suggesting a chain composition of $[\alpha 1(II)]_3$. By contrast the collagen produced by the cultured chondrocytes contained both α1 and α2 chains with a varying chain ratio (α1:α2 usually 2:1; occasionally as high as 4:1). The articular chondrocytes under these conditions of culture thus apparently produced a collagen resembling that of skin and cultured cutaneous fibrocytes having a chain composition $[\alpha 1(I)]_2\alpha 2$.

3) Because articular cartilage is an avascular tissue, having a predominantly glycolytic metabolism, anaerobic conditions are frequently cited as a force for chondrogenic differentiation of mesenchyme. Proliferation of articular chondrocytes was unaffected by lowering the oxygen tension of the environment to 7% while growth of fibrocytes was reduced 20%. Cell for cell the chondrocytes had higher rates of aerobic and anaerobic glycolysis than did the fibrocytes. Both cell types displayed a Pasteur effect accompanied by an increase in the specific activity of several glycolytic and also by Krebs cycle enzymes. Nevertheless, reduction of the oxygen tension did not increase sulfated mucopolysaccharide synthesis except, perhaps, at very low O_2 tensions (0.6%).

4) Rumalon, a proprietary extract of cartilage and bone marrow, has been widely employed, particularly in Europe, in the treatment of degenerative joint disease. This form of therapy has been based on a reported sulfation factor-like activity. No effect of rumalon was found either on DNA synthesis or radiosulfate incorporation by chondrocytes in monolayer culture. This was true whether or not the medium contained serum.

5) The coefficient of friction obtained in a glass-rubber bearing system rose, when either bovine or human synovial fluid was diluted with physiological salt solutions, to levels above those obtained with the saline solution alone. This bore no direct relationship to the dynamic viscosity of the diluted synovial fluids and indicates that under certain conditions, molecular changes in the synovial fluid (presumably its mucin) can cause it to bind rather than lubricate joints.

6) The anatomic findings in previous projects have been extended.

   A. Histologic studies performed between April 7, 1971 and April 20, 1972, in addition to 7 necropsies on patients with rheumatic disease, include:

Joint	Skin	Muscle	Other
72	33	4	58

B. The role of fat embolism as the principal pathogenetic factor in idiopathic or steroid-induced aseptic necrosis is being studied. Marchi preparations are being made in an attempt to overcome technical difficulties inherent in conventional frozen section techniques on the adipose tissues. Eight such cases are presently in various stages of preparation along with appropriate control cases.

Proposed Course:

We anticipate completion of the chemical analysis of the mucopolysaccharides of the various cultures by Dr. Srivastava, a visiting fellow who will stay on for an additional year. Further characterization of the "chondrocyte growth factor" that contaminates several glycoprotein hormones will be carried out. A new clinical associate with experience in electron microscopy, will study the amianthoid degeneration of costal cartilage. A guest worker, Dr. John Bland, will complete an anatomical study of the cervical spine in rheumatoid arthritis on which he and Dr. Sokoloff have collaborated sporadically over several years.

Publications:

Corvol, M.T., Malemud, C.J., and Sokoloff, L.: A pituitary growth-promoting factor for articular chondrocytes in monolayer culture. Endocrinology. 90: 262-271, 1972

Layman, D.L., Sokoloff, L., and Miller, E.J.: Collagen synthesis by articular chondrocytes in monolayer culture. Exper. Cell Res. In press

Malemud, C.J., and Sokoloff, L.: Failure of Rumalon to increase sulfate incorporation by articular chondrocytes in monolayer culture. Arthritis Rheum. 14: 779-780, 1971

Malemud, C.J., and Sokoloff, L.: Rumalon and serum-free culture of chondrocytes. Arthritis Rheum. In press

McCutchen, C.W.: Ghost wakes caused by aerial vortices. Weather, 1972

McDowell, R.S., and McCutchen, C.W.: The Thoreau-Reynolds ridge, a lost and found phenomenon. Science. 172: 973, 1971

Simon, W.H.: Scale effects in animal joints. II. Thickness and elasticity in the deformability of articular cartilage. Arthritis Rheum. 14: 493-502, 1971

Simon, W.H.: Wear properties of articular cartilage in vitro. J. Biomechan. 4: 379-389, 1971

Simon, W.H., and Green, W.T., Jr.: Experimental production of cartilage necrosis by cold injury: failure to cause degenerative joint disease. Amer. J. Path. 64: 145-153, 1971

Sokoloff, L.: The pathology of rheumatoid arthritis and allied disorders. In Hollander, J.L. (Ed.): Arthritis and Allied Conditions (ed. 8). Philadelphia, Lea and Febiger. In press

Sokoloff, L.: The pathology and pathogenesis of osteoarthritis. In Hollander, J.L. (Ed.): Arthritis and Allied Conditions (ed. 8). Philadelphia, Lea and Febiger. In press

Serial No. NIAMD-LEP-9
1. Experimental Pathology
2. Molecular Pathology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Protein and Proteolytic Enzyme Interrelationships in Normal and Disease States

Previous Serial Number: NIAMD-LEP-14

Principal Investigator: George G. Glenner, M.D.

Other Investigators: C. Isersky, Ph.D., Visiting Associate (July 1, 1971-Sept. 1, 1971), S. Kimura, M.D., Visiting Associate (July 1, 1971-July 1, 1972), D. Zopf, M.D., Clinical Associate in Pathology (July 1, 1971-May 3, 1972)

Cooperating Units: M. Harada, Ph.D. (Aichi-Gakuin University, Nagoya,Japan)
H. Bladen, Ph.D. (NIDR-LHP)
D. Eanes, Ph.D. (NIDR-LHP)
W. Terry, M.D. (NCI-I)
D. Ein, M.D. (NCI-I)

Man Years:

Total:	6
Professional:	4
Other:	2

Project Description:

Amyloidosis

Human Amyloidosis of Immunoglobulin Origin: Evidence obtained by amino acid sequence and immunochemical studies of purified amyloid fibril proteins suggests that amyloid fibrils consist primarily of the amino-terminal variable segment of the light polypeptide chain of homogeneous immunoglobulin in those cases studies by us (Drs. Glenner, Kimura, Isersky, Page, Harada, Zopf, Terry). If indeed this is their source, then it should be possible to produce a variable region fragment having the characteristics of amyloid fibrils from homogeneous light chains.

A method for cleaving Bence Jones proteins into variable and constant fragments by proteolysis has been described by Solomon et al. We subjected three κ and two λ Bence Jones proteins having no polysaccharide constituents from patients not known to have amyloidosis to a 2 to 5 hour incubation in glycine buffer, pH 3.5, in the presence of pepsin at 37°C. During the incubation, precipitates formed with the two λ Bence Jones proteins. These

precipitates were centrifuged at 8000g for 1 hour in a refrigerated centrifuge, and a portion of the sediments was stained with alkaline Congo red, examined by polarization microscopy, and found to have a green birefringence. Electron microscopy by the negative staining method showed that the precipitates were composed of fibrils measuring from 70 to 80 Å in width and from 1000 to 2000 Å in length, and that they had the appearance of twisted doublet filaments characteristic of amyloid fibrils. The remaining sediments were throughly washed with 0.1N ammonium acetate buffer, pH 5.0, and lyophilized. Examination of both sediments by x-ray diffraction methods revealed a strong, sharp band at 4.75 Å and a moderately strong diffuse halo at 9.3 Å. This picture is typical of the antiparallel β-pleated sheet configuration of amyloid fibrils. Infrared studies confirmed this x-ray diffraction interpretation of the fibril conformation. One of the λ Bence Jones proteins was chosen for further study. The proteolytically derived sediment (BJ fragment) was denatured in 6M guanidine hydrochloride buffer to pH 8.0 containing mercaptoethanol, exhaustively dialyzed with distilled water, and lyophilized. The molecular weight of the BJ fragment, as determined by column chromatography on Sephadex-G-100 equilibrated with 5M quanidine in 1N acetic acid, was 4600. This protein was reduced and aminoethylated. The aminoethylated protein was digested with tryspin, and peptide mapping was performed. Common region peptides from the peptide map of the BJ fragment were absent. The amino acid sequence of the aminoethylated Nic fragment was determined with an automatic amino acid sequencer. The sequence data in conjunction with peptide mapping and molecular weight studies showed that the BJ fragment derives exclusively from a portion of the variable region of BJ protein. These studies demonstrate that fibrils having the tinctorial, ultrastructural, and crystallographic properties of amyloid fibrils can be created from some but not all Bence Jones proteins by peptic digestion at pH 3.5 and 37°C. The fact that "amyloid" fibrils can be created from Bence Jones proteins at a physiologic temperature in the presence of a proteolytic enzyme having an acidic pH optimum suggests that one possible pathogenetic mechanism for amyloid formation may be by means of intralysosomal catheptic digestion of light polypeptide chains of immunoglobulins. This mechanism is supported by the frequent close spatial relationship between amyloid deposits and cells of the macrophage system and by the selection microscopic observation of fibrils within plasmalemmal invaginations and membrane-bound vesicle of macrophage. (Drs. Glenner, Terry, Ein, Eanes, and Bladen).

Human Amyloidosis of Unknown Origin: Further chemical analysis has shown that not all amyloid fibrils are derived from immunoglobulin light chains. Our definition of amyloid is based on certain derivative properties. It is a material deposited in tissues which exhibits Congo red polarization birefringence, has a fibrillar appearance by electron microscopy and characteristic x-ray diffraction pattern indicating a β-pleated sheet conformation. Under appropriated conditions, proteins other than light chains could assume these same properties. An anti-parallel chain β-pleated sheet conformation has been found by infra-red studies in the Fd fragment of immunoglobulin heavy chains. Studies of amino acid polymers show that poly-l-lysine in its β-conformation has many of the properties of amyloid

fibrils except for some difference in the spacing of the polypeptide chains of the β-sheet. Partial amino acid sequence determination of amyloid fibrils from one patient with classic "secondary" amyloidosis does not correspond,as of this writing, to any known protein sequence. Thus, some cases of amyloidosis may result from tissue deposition of portions of immunoglobulins other than light chains or of proteins other than immunoglobulins. (Drs. Glenner. Terry, Ein, Eanes, and Bladen).

Honors and Awards: President, Histochemical Society, 1971-72.

Publications:

Kerwar, S.S., Weissbach, Herbert, and Glenner, George G.: An amino-peptidase activity associated with brain ribosomes. Arch. Biochem. Biophys. 143: 336-337, 1971.

Glenner. G.G., Ein, D., Eanes, E.D., Bladen, H.A., Terry, W., Page, D.: The creation of "amyloid" fibrils from Bence Jones proteins in vitro. Science 174: 712-714, 1971.

Isersky, Chaviva, Page, David L. Cuatrecasas, Pedro, DeLellis, Ronald A., and Glenner, George G.: Murine amyloidosis: Immunologic characterization of amyloid fibril protein. J. Immunol. 107: 1690-1698, 1971.

Ein, D., Kimura, S., and Glenner, G.G.: An amyloid fibril protein of unknown origin: partial amino-acid sequence analysis. Biochem. Biophys. Res. Commun. 46: 498-500, 1972.

Isersky, C., Ein, D., Page, D.L., Harada, M., and Glenner, G.G.: Immunochemical cross-reactions of human amyloid proteins with human immunoglobulin light polypeptide chains. J. Immunol. 108: 486-492, 1972.

Glenner, George, G., M.D., Ein, Daniel, M.D., Terry, William D., M.D.: The immunoglobulin origin of amyloid. Amer. J. Medicine. 52: 141-147, 1972.

Serial No. NIAMD-LEP-10
1. Experimental Pathology
2. Molecular Pathology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Cytogenetics

Previous Serial Number: 15

Principal Investigator: Dr. J. H. Tjio

Other Investigator: Dr. B. J. White

Cooperating Units: Maryland Psychiatric Research Center
Dr. G. T. Ross (NICHD-RR)
Dr. R. Sherins (NICHD-RR)

Man Years:

Total:	2
Professional:	1
Other:	1

Project Description:

Cytogenetic Studies on the Effect of LSD: We are continuing a double-blind, controlled study on the effects on human chromosomes of LSD and DPT, hallucinogens which are administered to patients as part of their treatment at the Maryland Psychiatric Research Center in Baltimore. Analysis of the data is nearly finished, but no further results are available since the coded blood samples received from the patients have not yet been identified to us. Completion of the project has been delayed following the death of the principal collaborator (Dr. W. N. Pahnke) last year.

Chromosome Identification and Studies of Chromosomal Abnormalities in the Mouse and Human Using the Differential Staining Techniques of Quinacrine Mustard and Giemsa-banding.

Banding Patterns in Lymphocytic Cell Lines: Dr. Arthur Bloom (University of Michigan, Ann Arbor) has established 52 lymphocytic cell lines and 30 clonal sublines. The chromosomes of these lines have all been screened periodically to study the evolution of the karyotype within the lines. Virtually all the lines have more than 80% of the cells with 46 chromosomes. The increased resolution provided by the Giemsa-banding technique, however, necessitates a re-examination of the selection which may operate in these cultures, in terms of small, previously undetected translocations and inversions.

Dr. Bloom will choose a small number of cell lines for close examination in our laboratory with the Giemsa-banding technique. It will be interesting to analyze lines which are apparently normal by usual cytogenetic procedures but which have undergone functional changes, as, for example, in their patterns of immunoglobulin synthesis.

Studies of Mouse Chromosomes using Differential Staining Techniques: Normal mouse chromosomes from several strains and translocation chromosomes T1Wh, T163H, and T1A1d are being studied using the quinacrine mustard and Giemsa-banding methods. Previously, only the smallest autosome (number 19) and the Y chromosome could be identified, but with the new techniques, the specific chromosomes involved in the three translocations can be determined. Identification of specific chromosomes with known mouse linkage groups is then possible. The results of these studies are included in manuscripts concerning the studies of the mouse hybrids. For investigators attempting human linkage studies with the mouse-human cell hybridization technique, the Giemsa-banding method may enable better differentiation of human and mouse chromosomes in cell lines where it is hoped that only a single human chromosome remains.

Clinical and Cytogenetic Studies on Human Congenital and Developmental Disorders: The differential staining methods (Quinacrine mustard and Giemsa-banding) are now being applied in our laboratory to studies of patients with various genetic disorders, abnormalities of gonadal development and function and selected cases of infertility in the male. Since no gross chromosomal abnormalities are detectable using routine cytogenetic methods in many such patients, the differential staining techniques may provide a means to detect small structural changes in chromosomes. For example, altered banding patterns in one or more chromosomes could indicate a rearrangement not producing obvious changes in chromosome morphology, such as peri-centric inversion, some cases of reciprocal translocation, or duplication and deletion of chromosomal segments. Since the Y chromosome has a characteristic intensely fluorescent region with Quinacrine mustard staining and the X chromosome a characteristic Giemsa-banding pattern, differential-staining analysis of these chromosomes in patients with abnormal gonadal development and function may help us to determine the etiology of some of these problems.

Of special interest will be studies of blood smears, ejaculate, and meiotic chromosomes with quinacrine mustard from infertile males. The fluorescent Y-body of these cells will be studied; the finding of double Y-bodies in sperm reflect nondisjunction and is reported in the literature to be present in 1-2% of sperm of normals. Its incidence in infertile patients and normals will be evaluated (in cooperation with Dr. Richard Sherins, NICHD-RR).

Honors and Awards: None

Publications:

White, Beverly J., Van de Water, Lisa C., and Tjio, Joe-Hin: A family with balanced translocation, t(5p-;Gp+). J. Medical Genetics 8: 188-194, 1971.

Kamada, N., Morishima, A., and Tjio, J. H.: Blastogenesis of human peripheral lymphocytes: Studies on the mitotic cycle and the generation time by the use of tritiated thymidine. Clinical Immunology (Japan) 2: 657-665, 1971.

Serial No. NIAMD-LEP-11
1. Experimental Pathology
2. Molecular Pathology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Cytogenetic Studies

Previous Serial Number: NIAMD-LEP-16

Principal Investigator: Dr. B. J. White

Other Investigators: Dr. J. H. Tjio

Cooperating Units: Dr. E. Driscoll (NIDR-OMS)
Dr. R. Sherins (NICHD-RR)
Dr. J. Vaitukaitis (NICHD-RR)

Man Years:

Total:	2
Professional:	1
Other:	1

Project Description:

Cytogenetic and Reproductive Studies of Mice with Robertsonian Translocations and their Hybrids.

Continued Studies of the T1Wh Strain: This strain, developed in our laboratory in 1967, is now in generation 19 of inbreeding. Characteristics of the strain are unchanged, and the average litter size at birth has remained stable (6.2). Establishment of genetic homogeneity in this strain will facilitate our studies of developmental anomalies in aneuploid progeny of F_1 hybrids from crosses of T1Wh with other established translocation strains.

Meiotic and Other Studies of Mouse Hybrids: F_1 hybrids from crosses between T1Wh and T163H translocation homozygotes were studied. Analysis of meiotic chromosomes of the F_1 males showed abnormal meiotic pairing (quadrivalent formation) comparable to that found in humans carrying reciprocal translocations, as well as cytological evidence of regular non-disjunction for chromosome 19 (the smallest mouse autosome). A high incidence of mice trisomic for chromosome 19 were found among the progeny of the F_1 when crossed with each other (12% at birth, 11% of fetuses), with the parent strains (7.7% to 13.8% of fetuses), and with normal non-translocation-bearing mice (17.5% of fetuses). Some trisomics had cleft palates, and all of those studied after birth were smaller than their littermates and died during the first day of life. Even without cleft palate, such mice were unable to feed;

death usually resulted from respiratory distress or dehydration.

At present, trisomic fetuses are being compared to their normal littermates for the following: (1) total body weight, (2) weight of brain, lungs, and kidneys, (3) placental weight, and (4) gross and microscopic evidence of abnormal development. Of interest is the use of the trisomy 19 model to analyze the effects of autosomal imbalance on general growth and development as well as development of specific organs. Since chromosomal imbalance in the human is often associated with infertility, microscopic studies of the gonads of newborn mice will be of special interest. Likewise, comparison of the gross and microscopic characteristics of trisomic and normal fetal mouse brain may provide a model for understanding the basis of the profound effects of autosomal imbalance on human brain development and function. To date, our weight data indicate that, in trisomy 19, growth retardation begins soon after implantation and that the brain and other specific organs are uniformly reduced in size and weight. Microscopic and placental weight studies are not completed. To our knowledge, this system is the first example described in mammals in which trisomy for a specific autosome can be regularly produced. In addition to its value as a model to study how chromosomal imbalance produces anomalies in the human, it also is a system where the incidence of nondisjunction has been directly evaluated (cytologically in the F_1 hybrid, and in specific crosses) and where the effects of such factors as age, parity, and sex on nondisjunction can be tested. A manuscript concerning the development of this model has been submitted for publication.

F_1 hybrids from crosses between mice homozygous for T1Wh and T1A1d have also been studied. These hybrids also have an increased incidence of nondisjunction, but only one trisomic (chromosome 19) has been detected among their progeny at birth. Studies initiated last year to derive a strain of mice homozygous for both T1Wh and T1A1d with a reduced chromosome number of 36 (normal number = 40) from the F_1 hybrids have been completed. To date, the new strain has been fertile and shown no evidence of nondisjunction; average size of the first 10 litters was 6.1. The accumulation of translocations of the centric fusion (Robertsonian) type, with reduction of chromosome number by one for every added translocation chromosome (and maintenance of a constant number of chromosome arms), has long been considered as a possible evolutionary mechanism. Presumably, this rearrangement in chromosomal material would be accompanied by progressive change in the animal. Starting with a mixed colony of mice with normal chromosome number (40) and chromosome number of 39, the T1Wh strain with chromosome number of 38 was established in 1967 after crossing only the animals with 39 chromosomes. This past year, the addition of 2 more translocation chromosomes (T1A1d/T1A1d) has resulted in a still lower chromosome number (36) without any obvious deleterious effects. Further studies to compare the parent strains 38, (TW/TW and TA/TA) with the new strain 36 (TW/TW TA/TA) need to be done in order to test the significance of our breeding experiments with regard to specific evolutionary theory. However, this new system to produce such changes in the laboratory provides an opportunity to directly study the problem.

Cytogenetic and Dermatoglyphic Studies of Patients: Chromosomal studies on a mosaic patient with a cell line monsomic for a G chromosome have been completed. In this case, low-grade (12-15% of marrow and peripheral blood mitoses) mosaicism was associated with chronic idiopathic lymphedema, lymphocytopenia, and oligospermia. The meiotic chromosomes were normal, however. No chromosomal abnormalities were found among family members, and the patient's clinical status has been stable. Analysis of dermatoglyphs of immediate family members is not completed. Follow-up of this unusual patient will be of interest, since some individuals with abnormal cell lines have an increased risk of leukemia.

Dermatoglyphic and routine chromosomal studies of a child with Russel-Silver dwarfism and his family have been completed. Since the dermatoglyphic studies suggested the presence of chromosomal abnormality and none was detected using routine methods, chromosomes of this family are now being studied using the Giemsa-banding technique. These studies are being included in a case report along with other detailed studies of the patient.

Chromosomal and dermatoglyphic studies of gonadal dysgenesis patients are in progress in cooperation with other physicians (RR-NICHD). In order to define specific dermatoglyphic features of certain groups of patients, a comparison of dermatoglyphic features of individuals with the following conditions has been initiated: (1) precocious puberty, (2) failure of gonadal development and normal karyotype, (3) gonadal deficiency secondary to hypogonadotropism, (4) gonadal dysgenesis with 45,XO karyotype, and (5) gonadal dysgenesis with mosaicism. Dermatoglyphs from all of these types of patients have been collected, but the analyses will not be significant until a large series has been completed. Various tissues from some of these patients are being karyotyped by our laboratory, since presence and extent of mosaicism are of interest in the clinical evaluation of the patients as well as for purposes of the dermatoglyphic study.

In cooperation with Dr. R. Sherins, (RR-NICHD), meiotic chromosomes, blood smears, and ejaculates of males with abnormal sperm morphology and motility are being studied in our laboratory with the recently-developed differential staining methods. These studies will occasionally include autoradiographic analysis of DNA synthesis of meiotic cells from testicular biopsies (done as part of diagnostic evaluation for infertility) by means of short-term in vitro incubation with H^3-thymidine. By adding these more precise means of studying the meiotic process, it is hoped that more can be learned about the etiology of infertility in selected cases where no cause is apparent.

Cytogenetic Studies on the Effect of Diazepam (Valium) on Human Chromosomes: The Anesthesiology Section, OMA, NIDR is giving normal healthy patients Valium intravenously (IV) while conducting a study comparing the effectiveness of that agent and Nitrous Oxide for psychosedation in oral surgery. Analysis of conflicting reports of chromosome studies in the literature indicate the drug does not produce chromosome damage in vitro or when given to humans orally. However, further studies are in progress because the

the chromosome effects of this widely-used drug have not been studied after IV administration.

Our laboratory is receiving 5 blood samples from approximately 20 patients in the Valium study; samples before and after the drug is given are being analyzed for chromosome damage. All analyses are done blindly. This study is still in progress, and results are not yet available.

Studies of the Relationship of Chromosomal Abnormalities and Certain Viral Infections in Mice.

During the past year, this project has not been pursued. This is due to concentration by the other investigators on virus purification and DNA characterization. The studies of the relationship of MVM (minute virus of mice) to mouse chromosomes and its effect upon meiotic chromosomes of animals infected in utero will be continued after the other aspects of the study are completed.

Honors and Awards: none

Publications:

White, Beverly J., Van de Water, Lisa C., and Tjio, Joe-Hin: A family with balanced translocation, t(5p-;Gp+). J. Medical Genetics 8: 188-194, 1971.

ANNUAL REPORT
LABORATORY OF CHEMICAL BIOLOGY
NIAMD

The major research activities of the Laboratory of Chemical Biology continue to involve the investigation of structure-function relationships in macromolecules, the mechanisms underlying the folding of polypeptide chains to form proteins, and the nature of regulatory mechanisms in protein biosynthesis.

I. Studies on the relationship between amino acid sequences of polypeptide chains and the formation of functional three-dimensional protein molecules

Previous studies in the laboratory on the model protein, staphylococcal nuclease, have established that most of the amino acids in the sequence of the polypeptide chain are required to furnish the information necessary to determine the unique, functionally active structure of this enzyme. It has also been shown that certain points in the chain may be ruptured by chemical or enzymic cleavage to yield fragments that are capable of *in vitro* complementation, with the production of active, non-covalently bonded complexes. For example, a complex composed of residues 6-48 and 50-149 is relatively stable and exhibits about 10% of the activity of the native enzyme. The smaller of the two fragments has now been tagged with radioactive acetyl groups and study of the exchange of the tagged peptide with normal, unlabelled peptide has given information about factors responsible for the stability of the complex. The structures of the two separate peptide fragments are disordered. The major stabilizing force in the complex appears to be involved with suppression of unfolding of the native structure: that is to say, the dissociation of the complex is the rate-limiting step in the exchange.

Related studies were carried out on bovine pancreatic ribonuclease. A number of derivatives of this protein, lacking amino acid residues at one or the other end of the chain, were examined for their ability to undergo reversible reduction and reformation of disulfide bonds. It was possible to show in a more definitive way than earlier studies had suggested that residues both at the carboxyl and amino terminal ends are essential to the formation of correct disulfide bonds. These studies also support the concept that there is very little superfluous material in the fabric of protein molecules that is not essential for the determination of three-dimensional geometry. (Taniuchi, Bohnert, Anfinsen)

II. X-ray studies on the three-dimensional structure of Nuclease-T', an enzymically active derivative formed by complementation of two fragments of staphylococcal nuclease

Previous attempts to crystallize Nuclease-T (residues 6-48 + residues 49-149) in a form suitable for X-ray crystallographic work were unsuccessful. In the present experiments, it was shown that good crystals could be obtained if a contaminating peptide containing residues 50-149 was removed prior to addition to the smaller fragment, 6-48. Nuclease-T' was reconstituted by mixing of Nuclease-T-(6-48) and Nuclease-(49-149) at approximately equimolar ratio. Unbound, excess fragments were removed by digestion with

trypsin in the presence of pdTp and Ca^{++}. Nuclease-T' was purified by phosphocellulose column chromatography and crystallized in 0.0105 potassium phosphate, pH 8.3, with approximately 30% 2-methyl-2,4-pentanediol in the presence of pdTp and Ca^{++} at 4 to 5°. Nuclease-T containing Nuclease-T-(6-48) and Nuclease-T-(49-149) was also isolated from the limited digest of nuclease and similarly crystalized. The size of both Nuclease-T' and Nuclease-T crystals ranged up to 2.0 x 0.4 x 0.4 mm. X-ray precession photographs of Nuclease-T' crystals mounted in sealed glass capillaries were taken with a Nonius camera with Ni filtered CuK_{α} radiation from an Elliot X-ray generator at 4 to 5°. The crystals of Nuclease-T' and of Nuclease-T are also isomorphous. We conclude that the three-dimensional structure of Nuclease-T and Nuclease-T' very closely resemble that of native nuclease. The availability of Nuclease-T crystals suitable for X-ray crystallographic studies now makes it feasible to carry out direct studies of the three-dimensional structures of chemically synthetic and semisynthetic complexes and a variety of analogs of these materials in a study of the molecular basis of enzymatic function. (Taniuchi, Anfinsen, Davies).

III. The use of the organic synthesis of semisynthetic nuclease derivatives in the elucidation of mechanisms of enzymic function.

The main objective of this work has been to study which amino acid residues in staphylococcal nuclease-T define the proper structure and function of this enzymic species. A further objective of this work has been to correlate the results with the known three-dimensional structure of nuclease, and thus to implicate certain interactions in the folding of the polypeptide chain into the active conformation. The basic method employed has been Merrifield's method of solid phase peptide synthesis of the smaller fragment of nuclease-T. Peptides have been prepared possessing the sequence 6-47, with substitutions of different amino acid residues at specific points along the sequence. In addition, peptides have been prepared with truncations at the N-terminus and at the C-terminus. These peptides were assayed for their capacity to bind native fragemnts 49-149 and to generate DNase and RNase activity. The results have been compared with predictions based on the three-dimensional model of nuclease. The synthesis of fragments with truncations at the C-terminus has shown that residues 45 through 48 are not necessary for the formation of an active complex. Loss of residue threonine 44 brings about total loss of activity, but binding remains excellent. Becuase a synthetic peptide with the sequence 9-44 but with alanine in place of threonine at position 44 also gives activity when combined with native 49-149, it seems that it is the peptide bond between residues glutamic acid 43 and threonine 44 that plays an important role in the proper orientation of the γ-carboxyl group of glutamic acid 43 with Ca^{++} at the active site of nuclease-T. Such a role is played through the neutralization of the α-carboxyl group of glutamic acid 43. The fact that the synthetic fragment 9-44, Ala 44 produces activity when incubated with native 49-149 indicates that the sequence 9-44 contains sufficient information for the formation and the stabilization of an active complex. This sequence represents a 20% reduction in the native sequence of residues, a significant finding also to be reflected in the reduced time of synthesis of future analogs. (Chaiken, Sanchez, Anfinsen)

In related work, semisynthetic nuclease-T and ribonuclease-S have been prepared specifically for the study of factors concerned with the conformational properties of these two protein systems. Analogs have been made containing ^{13}C. For semisynthetic nuclease-T', experiments have been completed on an active site analog containing aspartic acid at position 43 in place of the normally occurring glutamic acid. [Asp 43]-Semisynthetic nuclease-T' has an overall conformation similar to that of native nuclease-T' but lacks enzymic activity. Nonetheless, the complex does bind the active site ligands Ca^{++} and deoxythymidine-3',5'-diphosphate, thus defining the critical role glutamic acid 43 as a direct participant per se in catalysis by nuclease-T', and perhaps for that by intact nuclease as well. The isolation of other semisynthetic nuclease-T' analogues is in progress. Analogs of semisynthetic ribonuclease-S' have been made for active site and conformationally important residues in the (1-15) fragment. In one set of experiments, ^{13}C-enriched and ^{19}F-containing synthetic-(1-15) peptides have been prepared and used to study the interaction of the (1-15) fragments with ribonuclease-(21-124) by carbon 13 and fluorine 19 nuclear magnetic resonance. It has been shown that such selective enrichment can be achieved synthetically and that the products provide valuable tools for measuring structural parameters for specific residues in going from disordered (denatured) to ordered (native) environments. The techniques involved also have indicated the application to the study of the mechanism of action of ribonuclease A and ribonuclease S. (Chaiken, Sanchez, Griffin, Cohen)

Of the four histidine C-2 proton NMR peaks of nuclease only that of His 124 is assigned. Nuclease-T comprising fragments 6-48 and 49,50-149 has enzymic activity, has been crystallized, and, from the X-ray diffraction pattern, has been shown to be highly isomorphous with nuclease. It is thus probable that the conformations of nuclease-T in solution include those of nuclease. The following peptides have been made by the solid phase method: 6-47 His 46,Gly 8; 6-47 Gly 46,His 8; 6-47 Gly 46,Gly 8; 6-47 His 46,His 8 (the native sequence); and have enzymic activity on complementation with 49-149. When conditions in which nuclease-T gives a nuclease-like NMR spectrum have been found, the combination of the above fragments with native 49-149 will enable us to uniquely assign the C-2 proton resonances to their respective histidines. (East, Schechter, Anfinsen)

Finally, in connection with the use of organic synthetic methods in the laboratory's research program, efforts have been made to improve the speed and efficiency of the Merrifield solid phase synthetic procedure. Methods were developed for following the coupling and deblocking reactions during solid-phase synthesis in small funnels so that the time course could be determined. An apparatus was constructed permitting the rapid addition of reagents with mixing by bubbling rather than by rocking. The process of shrinking and swelling the resin in order to increase washing efficiency was studied and employed. Both the coupling and deblocking reactions were found to proceed much faster than was originally anticipated. In addition, the loss of peptide from the resin was found to be linear with time of exposure to TFA. Therefore, it seemed that much faster reaction times would be advantageous. Washing by shrinking and swelling was found to be superior to the standard washing techniques. Using these modifications, the solid-phase synthesis of bradykinin, a naturally occurring nonapeptide, was performed

in less than five hours. The material was fully active by a biologic assay. (Sachs, Corley, Anfinsen)

IV. Studies on the mechanism of action of staphylococcal nuclease

The variation of the kinetic parameters with pH has allowed the determination of the ionization constants of functional groups of the protein involved in substrate binding and catalysis. This data will aid in the further elucidation of the identity of these critical residues. The comparison of the pH-independent plateau rates for reactions in H_2O and D_2O has revealed the lack of a significant solvent isotope effect. This result will place restrictions on the mechanistic possibilities and will aid in elucidation of the mode of catalysis. Examination of the incorporation of ^{18}O from solvent water has confirmed the cleavage position as occurring between phosphorous and oxygen. This information will also aid in delineating the mechanism. Under a variety of conditions, the inhibition by pdTp has yielded complex, non-linear effects, suggesting strongly that multiple binding to the enzyme is occurring. Attempts to quantitate this phenomenon using equilibrium dialysis have met with limited success but the synthesis of inhibitor with higher specific activity will aid in this goal. Woodwards reagent K, known to react specifically with carboxylic acid groups, provides a rapid and complete inactivation of the enzyme. The reaction has been characterized with respect to rate variation with pH, the influence of added calcium plus pdTp, reagent K concentration, enzyme concentration, and the influence of the degradative hydrolysis of the reagent. The observation of a kinetically significant ionization constant in this process will allow the assignment of this pK_a to a functional residue perhaps involved in the maintenance of essential tertiary structure. (Dunn, Chaiken, DiBello, Anfinsen)

V. Studies on the conformational stability of proteins, and the effects of ligands using nuclear magnetic resonance and fluorescence spectroscopy

Two powerful tools, nuclear magnetic resonance for the examination of thermodynamic aspects of protein conformation and fluorescence spectroscopy for the study of the kinetics of protein folding and unfolding, have been applied to staphylococcal nuclease, ribonuclease, myoglobin, and cytochrome c. The Varian 220 MHz nuclear magnetic spectrometer with a probe for recording proton resonances has been used for most of our work. Protein samples have been exchanged with pure D_2O and residual protons have been measured and recorded on a Varian time averaging computer. pH titrations have been accomplished by measuring time-averaged spectra at closely spaced pH values. Temperature, ionic strength, and the concentration of substrate analogs have been used as independent variables. Selected chemical modifications of proteins have also been accomplished for the purpose of identifying certain resonances. The imidazole C2 protons of the four histidine residues of bovine pancreatic ribonuclease have been resolved from other resonances and titration curves of chemical shift vs. pH constructed. Only one of the four curves fits the Henderson-Hasselbalch titration curve. The other three show acid perturbations and one shows an alkaline perturbation as well. On the basis of previous studies of histidine model compound titration curves and mathematical models for interacting groups, these acid and alkaline

effects can be ascribed to the existence of neighboring titrating groups. These groups may be tentatively ascribed to lysine and aspartic acid or glutamic acid residues. The effects of substrate analogs, including uridine and cytidine monophosphates and a new class of nucleotide phosphonates, on these titration curves have been studied. The mononucleotides give effects which can be interpreted with reference to the base and the position of the phosphate. The dinucleotide phosphonate seems to be a good analog of the substrate and has allowed us to measure the pK values of the various protons in the active site and in the inhibitor and to make inferences about the geometry of the active site. Titration curves of chemical shift vs. pH have been constructed for the histidine residues of sperm whale and horse heart metmyoglobin. Comparisons of these have allowed the assignment of one particular histidine residue to a resonance. The effect of changing the state of ligation of the iron has been detected in the resonance of another histidine. Marked changes in these spectra also occur upon removal of the heme group or fragmentation of the enzyme. Cytochrome c has been studied in an analogous way and the pK of the one titrating histidine residue has been determined. The effects of various chemical and physical modifications of the protein have been studied. (Schechter, Griffin, Sachs, Fisher, Hagenmeier, Eastlake)

The acid-induced conformational change in staphylococcal nuclease has been measured by stopped flowed spectrophotofluorometry of the single tryptophanyl residue. The kinetics of the structural change have been studied as a function of temperature, pH, and ionic strength. Equilibrium aspects of this transition have also been measured by viscosity, circular dichroism, and NMRm methods. A mathematical analysis of the kinetic data is also continuing in order to describe the mechanism of folding of this protein. The effects of calcium and thymidine diphosphate on the resonances yielded by the 4 histidine C2 ring protons has been examined by high resolution NMR measurements. Parallel studies have been carried out using the lanthanides Eu^{+++} and La^{+++}. The effects induced by calcium ions and the more complicated effects due to the paramagnetic lanthanides are under continuing investigation. In general, these metal ions induce shifts in the pK values as a result of specific binding to the active site. Other effects resulting from the paramagnetic properties which lead to shifts in the positions of the histidine resonances are more complex and under investigation. The studies in general may be of value in understanding the relationships between small movements of atoms in the protein structure and the enzymic activity. (East, Schechter)

VI. Studies on polymeric protein systems

β-galactosidase of B. megaterium, as purified by affinity chromatography, was reported in last year's annual report, and has been studied from the standpoint of whether the monomer of this normally tetrameric molecule possessed enzyme activity. The isolated monomers were found to be completely inactive and partial reconstitution of activity could be obtained by adding free monomer to Sepharose-immobilized monomer. This suggests that the active site of the enzyme is a dimer in contrast to the enzyme as found in E. coli, where only the tetrameric form has activity. (Pollard, Steers)

Studies were also carried out to isolate and characterize the structural

proteins from trichocysts of Paramecium aurelia; to compare the proteins found in wild type with those found in various mutant strains; to gain an understanding of some of the processes associated with the development of this cellular organelle. The solubilization of trichocysts is salt dependent, as they are most readily solubilized by heating in the absence of any salts. At the present time, column chromatography and ultracentrifugation studies have produced data in which the former technique suggests a highly aggregative system while the latter technique does not. Further work is needed to clarify these and other observations before a more definitive characterization of trichocysts proteins is to be reported. Once this has been accomplished, an analysis of various mutants will be undertaken. This work represents a continuation of our objectives to characterize structural proteins related to the cell surface and cell surface properties using a specialized system in microorganisms. The analysis of genetic mutants will hopefully be useful in elucidating some of the problems of protein assembly into a cellular organelle. (Pollack, Steers)

VII. Studies on the regulation of histidine biosynthesis in Salmonella typhimurium

Studies on regulation of the histidine operon have continued to focus on the role of the first enzyme for histidine biosynthesis, the G enzyme, in controlling the rate at which the histidine operon is transcribed. The findings have been of four kinds:

1) The enzyme was previously shown to have an effect on repression of the histidine operon in vivo. Using various merodiploid strains, it was possible to show that this effect of the enzyme is carried out in a trans dominant fashion. Thus, the G enzyme exerts its effect on the repression process not by acting locally, at its site of synthesis, but by acting as a freely diffusible gene product, as expected for a regulatory protein such as a repressor. (Kovach, Vogel, Goldberger)

2) The enzyme was previously shown to have a high affinity for histidine tRNA. Using partially purified preparations of various species of tRNA, it was possible to demonstrate that the enzyme not only binds histidine tRNA in preference to tRNA aminoacylated with any other amino acid, but also that it binds the aminoacylated form of $tRNA^{His}$ in preference to the deacylated form of $tRNA^{His}$. This finding would be expected for any protein which interacts with histidyl-tRNA to form a regulatory complex, since the repression system is known to be responsive to the intracellular concentration of histidine tRNA only in the aminoacylated form. (Kovach, Vogel, Goldberger, Deeley)

3) If the G enzyme plays an obligatory role in regulation of the histidine operon, it should be possible to identify a class of mutants in which mutation of the gene specifying the G enzyme leads to loss of repression control. By using a positive selection technique for feedback resistant mutants, a mutant of this type has been identified. (Kovach, Goldberger)

4) If the G enzyme exerts its regulatory effect at the genetic level, it should interact directly with some regulatory elements in the DNA of the

histidine operon. This has been studied with the use of a phage carrying the histidine operon in place of some of its own genes (Ø80dhis). The G enzyme was found to bind specifically to the bacterial DNA carried in the phage genome. It is tempting to speculate that the site of binding is in a specific region of the histidine operon. (Kovach, Vogel, Goldberger, Levinthal, Bruni, Blasi)

VIII. Regulation of enzyme synthesis in mammalian cells in tissue culture

This new direction has been initiated to permit the study of repression of amino acid biosynthetic pathways in mammalian cells in tissue culture. In order to achieve the above objective, a tissue culture laboratory was constructed and equipped. The laboratory was set up so that tissues could be cultured either in culture dishes or in suspension. Six different mammalian liver cell lines were then screened for presence of several amino acid biosynthetic pathways using modified assay systems. In addition, several cell lines were adapted to growth in suspension culture in order to increase the cellular yield for enzyme isolations. Several cellular systems involving the metabolism of amino acids have been found suitable for further study. These systems will be used to study variation in enzyme level and activity with changes in nutrition, substrates, and growth conditions and to determine the mechanisms of these variations. (Lipson)

Serial No. NIAMD-LCB-1
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on the relationship between the amino acid sequences and the functional three-dimensional structures of staphylococcal nuclease and bovine pancreatic ribonuclease A: the mechanism of the specific folding of protein.

Previous Serial Number: NIAMD-LCB-1

Principal Investigator: Hiroshi Taniuchi

Other Investigators: Janice L. Bohnert
Generoso Andria

Man Years:

Total: 1.6
Professional: 0.5
Others: 1.1

Project Description:

Objectives: To find the mechanism of folding of protein on the basis of the amino acid sequence.

Methods Employed and Major Findings: As reported in the previous year, labelled Nuclease-T', ((6-48)+[1-^{14}C]acetyl-(50-149)) was prepared by complementation of two inactive disordered fragments, the fragment of residues 6 to 48, (6-48), and the labelled fragment of residues 50 to 149, [1-^{14}C]acetyl-(50-149), of staphylococcal nuclease. [1-^{14}C]acetyl-(50-149) was obtained from Nuclease-T, which had been subjected to limited acetylation at pH 5.8 with [1-^{14}C]acetic anhydride. The average population of [1-^{14}C]acetyl-(50-149) contained 3 acetyl groups. Enzymic activity of ((6-48)+[1-^{14}C]acetyl-(50-149)) is approximately 50% that of ((6-48)+(50-149)). The helical content of both the former and the latter complex is 13% by the measurement of reduced mean residue rotation at 233 nm, $[m']_{233}$, at pH 7.8 as a function of temperature. The standard free energy involving the binding of (6-48) to (50-149) and to [1-^{14}C]acetyl-(50-149) has been calculated in the range of the thermal transition of $[m']_{233}$ assuming "two state transition." The free energy-temperature function of Hearn et al. ((1971) Biochemistry, 10, 806) has been applied to obtain a quantitative relationship between the standard free energy of the binding of the two Nuclease-T fragments and the temperature. The parameters of the function have been obtained by the least square method with the aid of a computer (PDP 10). The thermodynamic parameters involving the formation of

Nuclease-T from the two fragments have been calculated on the basis of the relationship between standard free energy of the binding of the two fragments and the temperature. The values concerning the formation of ((6-48)+(50-149)) are; $\Delta F°$: -8.7 Cal $mole^{-1}$, ΔH: -48 Cal $mole^{-1}$, $\Delta S°$: -133 e.u. $mole^{-1}$ and those with (6-48)+[1-^{14}C]acetyl-(50-149)) are ; $\Delta F°$: -8.7 Cal $mole^{-1}$, ΔH: -52 Cal $mole^{-1}$, $\Delta S°$: -147 e.u. $mole^{-1}$. The value of $\Delta F°$ is consistent with that obtained independently by the measurement of enzymic activity generated upon the binding of (6-48) to (50-149) (-9.0 Cal $mole^{-1}$ at 25°). The results indicate the close similarity of structural and thermodynamic properties of ((6-48)+(50-149)) and ((6-48)+[1-^{14}C]acetyl-(50-149)).

When ((6-48)+[1-^{14}C]acetyl-(50-149)) is mixed with (50-149) at pH 8, the exchange between bound [1-^{14}C]acetyl-(50-149) and free (50-149) occurs without inhibition even at 4°. The radioactivity of ((6-48)+[1-^{14}C]acetyl-(50-149)) and of exchanged out [1-^{14}C]acetyl-(50-149) has been determined after separation by affinity chromatography on a Sepharose-thymidine diphosphate column of Cuatrecasas, Wilchek and Anfinsen ((1968) Proc. Nat. Acad. Sci. U.S.A. 61, 636). The distribution of the bound and free forms of [1-^{14}C]acetyl-(50-149) at an equilibrium state is consistent with that expected on the basis of mass law irrespective of temperature. The kinetic data of the exchange have been processed by McKay's first order law of isotopic exchange. The rate of the exchange is dependent on the concentration of ((6-48)+[1-^{14}C]acetyl-(50-149)) in the first order but independent of that of (50-149). The results are consistent with the mechanism in which the dissociation of ((6-48)+[1-^{14}C]acetyl-(50-149) is the rate limiting step of the exchange. The apparent rate constant of the dissociation of ((6-48)+[1-^{14}C]acetyl-(50-149)) have been calculated to be 3.0×10^{-5}, 7.8×10^{-5}, 1.7×10^{-4} and 6.2×10^{-4} sec^{-1} at 4, 10, 15 and 20°, respectively. The activation enthalpy, entropy and free energy of the dissociation of ((6-48)+[1-^{14}C]-(50-149)) have been found to be 31 Cal, 32 e.u. and 22 Cal (20°) respectively by the modified Arrhenius plot.

As described in previous years and in the current report of NIAMD-LCB-2, the structures of the two fragments, (6-48) and (50-149) (and (49-149)) are disordered but the structure of Nuclease-T' closely resembles that of intact nuclease. Therefore, the observations described above indicate that the three-dimensional structure of Nuclease-T' involves a dynamic equilibrium of folding and unfolding under physiological conditions. It has been also observed that the binding of thymidine diphosphate and Ca^{++} to ((6-48)+[1-^{14}C]acetyl-(50-149)) strongly suppresses the exchange out of [1-^{14}C]acetyl-(50-149). Therefore, it is suggested that the three-dimensional structures of nuclease also involves a dynamic equilibrium of folding and unfolding under physiological conditions and maintained mainly by suppression of the unfolding rather than by acceleration of the specific folding. As also reported in previous years, such thermodynamic force stabilizing the structure of nuclease is apparent only after the assembly of the amino acid sequence is nearly complete during biosynthesis.

Des-(121-124)-RNase and des-(119-124)-RNase, the derivatives of ribonuclease A lacking 4 and 6 carboxy-terminal residues, respectively, cannot form the correct disulfide bonds after reduction and reoxidation even in the presence of a complementing fragment, RNase-(105-124)(1969-1970 and 1970-1971, Serial No. NIAMD-LCB-1). Randomly oxidized des-(119-124)-RNase can be converted to that with correctly paired disulfide bonds by disulfide interchange only in

the presence of the fragment RNase-(105-124) (1970-1971, Serial No. NIAMD-LCB-1). The observations are interpreted as follows. Reduced des-(119-124)-RNase and the fragment RNase-(105-124) do not interact in a specific way to lead to the correct pairing of half cystine residues upon oxidation. But the fragment binds only to des-(119-124)-RNase having correct disulfide bonds which accounts for approximately 1% the population of randomly oxidized des-(119-124)-RNase. The complex thus formed is much more resistant against disulfide interchange than any other unbound derivatives. As soon as des-(119-124)-RNase having correct disulfide bonds is formed, by random disulfide interchange, from des-(119-124)-RNase with incorrectly paired disulfide bonds, the fragment RNase-(105-124) binds to the "native" form of des-(119-124)-RNase and stabilizes it against disulfide interchange. If this hypothesis is the case, it is also possible that reduced native RNase does not form correct disulfide bonds upon oxidation without disulfide interchange. The hypothesis is partly supported by the following observations. Venetianer and Straub (1964) Biochim. Biophys. Acta, 89, 189) and Givol et al. ((1964) J. Biol. Chem., 239, 3114) have showed that the rapid oxidation of reduced RNase by dehydroascorbic acid yields enzymically essentially inactive product which is converted to native RNase by disulfide interchange. We have examined the rapid oxidation product of reduced RNase by gel filtration on Sephadex G-50 column. It has been found that approximately 1% the product is the native form of RNase and the rest is enzymically inactive materials having the same molecular weight as that of RNase but showing a larger hydrodynamic volume than that of native RNase. Accordingly, it is suggested that the disulfide interchange may be an essential step in the formation of the structure of native RNase during biosynthesis. The removal of 4 carboxyl terminal residues may prevent the formation of the "Native" structure of the derivative resistant against disulfide interchange. Therefore, the loss of the 4 carboxy terminal residues becomes lethal to the mechanism of the formation of correct disulfide bonds after reduction and reoxidation described above.

The presence and absence of amide groups at 4 aspartic acid and 4 glutamic acid residues of the amino acid sequence of nuclease (Foggi strain) has been determined. These residues are glutamic acid 73, glutamic acid 75, aspartic acid 83, glutamic acid 129, glutamine 131, asparagine 138, aspartic acid 143, asparagine 144 and aspartic acid 146. The studies reported in previous years and the present information establish the amino acid sequence of stpahylococcal nuclease (Foggi strain). Edman degradation with the combination of the identification of 3-phenyl-2-thiohydantoin derivatives of amino acid by thin layer chromatography has been employed to determine specifically asparagine, aspartic acid, glutamine and glutamic acid residues.

Significance to Biomedical Research: The observations concerning the relationship between the amino acid sequences and the mechanism of folding of nuclease and RNase indicate that the unique structure of the native protein cannot form in a large population during biosynthesis until the assembly of the amino acid sequence is nearly complete. There may be no dominant population of any conformation of an incomplete polypeptide chain. The hydrodynamic volume of the incomplete polypeptide chain may be larger than that of the native protein. Therefore, the structure of the incomplete polypeptide chain

is disordered and contains solvent molecules in its domain. The addition of a few amino acid residues to the polypeptide chain near the end of the assembly of the amino acid sequence may produce the unique conformation of the native protein. The force to stabilize the native structure appears to work by suppressing the unfolding of the native conformation which may involve a dynamic equilibrium of folding and unfolding. Such mechanism of the folding of protein may be rather universal in the biological system. The nature of the force described above appears to be unique and different from any of those accounted for in the existing theoretical treatment of protein conformation. If this is the case, a fuller understanding of the nature of the force may be important. For, the biological system is crucially regulated through the conformational change of protein molecules.

Proposed Course of Project:

1) Study the kinetics and the thermodynamics involving the interaction between Nuclease-T-(6-48) and Nuclease-T-(50-149)(or Nuclease-T-(49-149)) to form Nuclease-T' and that between Nuclease-(1-126) and Nuclease-(111-149) to form a second complementing structure (1969-1970 Serial No. NIAMD-LCB-1).

2) Study the reduction and reoxidation of RNase A and its complementing system in order to establish the basic mechanism of correct pairing of disulfide bonds. A similar study may be extended to other proteins, e.g. lysozyme.

3) Study the crystallization of the complexes of the complementing systems of nuclease (other than Nuclease-T', see Serial No. NIAMD-LCB-2) and RNase A in order to examine the complementing structures by X-ray diffraction.

Publications:

Andria, G., Taniuchi, H., and in part Cone, J.L.: The specific binding of three fragments of staphylococcal nuclease. J. Biol. Chem. 246: 7421-7428, 1971.

Taniuchi, H.: Chemical and physical factors involved in protein folding as exemplified by staphylococcal nuclease, Part I. Equilibrium. PAABS Revista in press, 1972.

Bohnert, J.L. and Taniuchi, H.: The examination of the presence of amide groups in glutamic acid and aspartic acid residues of staphylococcal nuclease (Foggi strain). J. Biol. Chem. 247, in press, 1972.

Serial No. NIAMD-LCB-2
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: X-ray studies on the three-dimensional structure of Nuclease-T', an enzymically active derivative formed by complementation of two fragments of staphylococcal nuclease.

Previous Serial Number: LCB-NIAMD-2

Principal Investigators: Hiroshi Taniuchi
Christian B. Anfinsen

Cooperating Unit: David R. Davies (NIAMD, LMB)

Man Years:

Total: 0.8
Professional: 0.8

Project Description:

Objectives: To determine the three-dimensional structure of crystalline Nuclease-T' by X-ray diffraction.

Methods Employed and Major Findings: Two fragments of staphylococcal nuclease, Nuclease-T-(6-48) (residues 6 to 48) and Nuclease-T-(49-149) (residues 49 to 149) were prepared by limited digestion of nuclease with trypsin in the presence of thymidine-3',5'-diphosphate (pdTp) and Ca^{++} and purified as described in previous reports. Nuclease-T' was reconstituted by mixing of Nuclease-T-(6-48) and Nuclease-(49-149) at approximately equimolar ratio. Unbound, excess fragments were removed by digestion with trypsin in the presence of pdTp and Ca^{++}. Nuclease-T' was purified by phosphocellulose column chromatography and crystallized in 0.0105 potassium phosphate, pH 8.3, with approximately 30% 2-methyl-2,4-pentanediol in the presence of pdTp and Ca^{++} at 4 to 5°. Nuclease-T containing Nuclease-T-(6-48) and Nuclease-T-(49-149) was also isolated from the limited digest of nuclease and similarly crystalized. The size of both Nuclease-T' and Nuclease-T crystals ranged up to 2.0 x 0.4 x 0.4 mm. X-ray precession photographs of Nuclease-T' crystals mounted in sealed glass capillaries were taken with a Nonius camera with Ni filtered CuK_α radiation from an Elliot X-ray generator at 4 to 5°. The reciprocal lattice planes of (0kl) and (n0l) were identical and showed a tetragonal unit cell of dimensions a=b=47.6±0.2 A, c=63.3±0.2 A. The systematic extinction of 00l for $l \neq 4n$ demonstrated the space group to be $P4_1$, or its enantiomorph $P4_3$. Reflections observed were to spacings of 3A. The results indicate that the unit cell of Nuclease-T' is essentially identical with that reported for the crystalline liganded nuclease (Arnone et al. (1969) Proc. Nat. Acad. Sci. U.S.A.

64, 420). The high degree of isomorphism of Nuclease-T' and of nuclease is indicated by comparing the precession photograph of Nuclease-T' crystal with that of crystalline nuclease obtained by the same method described above. A similar X-ray examination has shown that the crystals of Nuclease-T' and of Nuclease-T are also isomorphous. We conclude that the three-dimensional structure of Nuclease-T and Nuclease-T' very closely resemble that of native nuclease.

Significance to Biomedical Research: As reported previously the three-dimensional structure of Nuclease-T in solution appears to resemble that of nuclease. However, the two fragments, Nuclease-T-(6-48) and Nuclease-T-(49-149), when examined alone, are enzymically inactive and structurally disordered but recombination of the two restores the ordered structure and enzymic activity of Nuclease-T. This complementing system has afforded an excellent test object for direct examinations of structure-function relationship in nuclease (reviewed in the publication by Anfinsen et al. listed below) and of the dynamic events involved in the formation of the three-dimensional structure of nuclease (see NIAMD-LCB-1). In order to interpret these experiments without ambiguity it is necessary to establish the relationship between the structures of Nuclease-T' and nuclease at the level of atomic resolution. Such a comparison might in addition help to explain the lower enzymic activity (approximately 8% that of native nuclease) of Nuclease-T (and Nuclease-T') and aid in understanding the mechanism of the enzymic activity of the native protein. The availability of Nuclease-T' crystals suitable for X-ray crystallographic study also gives feasibility to the direct study of the three-dimensional structure of chemically synthetic and semi-synthetic Nuclease-T' and of its analogues containing substituted residues.

Proposed Course of Project:

1) The elucidation of the three-dimensional structure of crystalline Nuclease-T' by making a difference electron density map of Nuclease-T' and nuclease at high resolution.

2) The determination of the phases of the structure factors of crystalline Nuclease-T'.

3) The crystallization and X-ray diffraction study of semi-synthetic Nuclease-T'.

Publications:

Anfinsen, C.B., Cuatrecasas, P., and Taniuchi, H.: Staphylococcal nuclease, chemical properties and catalysis. The Enzymes, Vol. 4, P.D. Boyer (Editor), 3rd ed., p. 177, Academic Press, New York, 1971.

Taniuchi, H., Davies, D.R., and Anfinsen, C.B.: A comparison of the X-ray diffraction patterns of crystals of reconstituted nuclease-T and of native staphylococcal nuclease. J. Biol. Chem., 247: in press, 1972.

Serial No. NIAMD-LCB-3
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The kinetic and equilibrium studies on the relationship between amino acid sequences and the formation of the functional structures of various proteins.

Previous Serial Number: None

Principal Investigators: Waldo Fisher
Theodore Peters, Jr.
Albert Light
Hanspaul Hagenmaier
Hiroshi Taniuchi
Christian B. Anfinsen

Man Years:

Total: 2.8
Professional: 2.8

Project Description:

The general objective of this project is to find the relationship between the amino acid sequence and the functional three-dimensional structure of protein. Under this general objective, the individual subjects have been studied independently and are described below under the name of the main worker in each area.

Waldo Fisher: The study of the mechanism of the folding of cytochrome c

Objective: To find whether apocytochrome c is folded into a conformation approaching the native structure of cytochrorome c in the absence of the prosthetic group or whether the covalently bonded heme plays a crucial role in stabilizing the native conformation of cytochrome c.

Methods Employed and Major Findings: Horse heart cytochrome c has been chemically modified in the following way. The iron has been removed from the porphyrin ring by the use of HF yielding "porphyrin cytochrome c." The two thioether bonds attaching the heme moiety to cytochrome c have been cleaved by the Ag_2SO_4 method forming "apocytochrome c." Currently, the conformations of these three molecules, cytochrome c, porphyrin cytochrome c and apocytochrome c in solution are being comparatively examined by the measurements of circular dichroism, viscosity, absorption spectrum and fluorescence. A preliminary

result indicates that the structure apocytochrome c is disordered and different from that of the compact native cytochrome c.

Significance to Biomedical Research: The native cytochrome c contains covalently bonded heme. However, when synthesized on the ribosome a heme-free protein is produced which then has the heme moiety inserted, presumably within mitochondria (Kadenbach, (1972) Eur. J. Biochem., 12, 392). It is not known whether the apoprotein has a structure which determines the orientation of the inserted heme moiety or whether the interaction between the apoprotein and the ligand is necessary to form the structure of cytochrome c. The examination of the structures of apocytochrome c and porphyrin cytochrome c as compared with that of the native cytochrome c would give the answer to these questions. The results of the present study may also provide a suggestion for the role of a specific enzyme, if it exists, to catalyze the covalent bonding of the heme moiety to the apoprotein to form the native cytochrome c.

Theodore Peters, Jr.: The study of the relationship between the amino acid sequence and the folding of albumin.

Objective: To apply the technique of affinity chromatography to the study of serum albumin, in order to permit identification and isolation of functional submolecular segments and to aid in deducing the mode of biosynthesis and folding of this protein.

Methods Employed and Major Findings: Large fragments of albumin have been prepared, using selective cleavage with cyanogen bromide or trypsin; these have been isolated by conventional techniques and assayed by thin layer Sephadex, cellulose acetate electrophoresis and amino acid analysis. Goat anti-albumins antibodies have been isolated on an albumin-Sepharose column and coupled to Sepharose for use as an immunoabsorbent. Four fragments have been obtained from bovine albumin, of molecular weights 46,000, 44,000, 22,000 and approximately 15,000. The larger two of these give a precipitin reaction; the smaller two do not give a precipitin reaction but have been shown to bind to the insolubilized antibody. Two other fragments of molecular weights 8500 and 2800, isolated after peptic digestion, have been found to be unreactive in both immunochemical systems.

Significance to Biomedical Research: The elucidation of the mechanism of folding of albumin may help us to understand the functional role of albumin.

Albert Light: The study of the kinetics of the folding of Nuclease-T.

Objective: To analyze factors involved in the folding of Nuclease-T by the kinetic method.

Methods Employed and Major Findings: The folding of the polypeptide chain to regenerate the properties and biological activity will be studied with two complementing tryptic fragments, Nuclease-T-(6-48) and Nuclease-T-(50-149)(see Serial No. NIAMD-LCB-1) of staphylococcal nuclease. The change in structure from a random (peptide structure) to an ordered state (nuclease-

T') will be followed with stopped-flow measurements of the fluorescent emission spectra of the single tryptophan of the protein. It is known from earlier studies that the emission spectra shifts in a wavelength and relative intensity as the molecule changes from the denatured state to a folded structure. Furthermore, the region of biological activity on complementation of the fragments is correlated with the changes in the fluorescent spectra of the tryptophan.

The rate of formation of nuclease-T' from the fragments Nuclease-T-(6-48) and Nuclease-T-(50-149) will be slower than the folding of a denatured molecule of intact nuclease. As a result of a relatively slow rate, the kinetics of assembly can be followed over a long time span. Consequently, both the initial rate and the rate at later periods can be obtained and compared. It should be possible to establish the kinetic order and the corresponding observed rate constants of the reaction for the entire assembly process. The kinetics will be examined at several pH values, particularly at the pH optima for stability and biological activity.

Kinetic measurements can also be obtained with temperature as a variable. Although limited range of temperatures may be allowed, it should be possible to obtain thermodynamic information on the folding process which could be compared to those derived from equilibrium measurements.

Significance to Biomedical Research: These studies may serve as a beginning to examine the similarities and differences which may exist with a series of other complementing fragments of staphylococcal nuclease. It will be of interest to know if the assembly time changes if the folded structure contains redundant regions. These studies will also establish the feasibility of examining other proteins in which complementation of fragments is possible to regenerate the three-dimensional structure and help to understand the dynamics of folding of protein.

Hanspaul Hagenmaier: The study of the relationship between the amino acid sequence and the folding of myoglobin.

Objective: To find the minimum covalent structure required for the formation of the three-dimensional structure of myoglobin.

Methods Employed and Major Findings: Horse myoglobin was subjected to limited digestion with trypsin after the ϵ-NH_2-groups of the lysine residues had been blocked by the use of citraconic anhydride. Since horse myoglobin contains only two arginine residues (residues 31 and 139), the limited digestion is supposed to yield three fragments of residues 1 to 31, residues 32 to 139 and residues 140 to 153. These three fragments, after deblocking, have been separated by gel filtration and identified by amino acid analysis and by the determination of amino and carboxyl termini. The large fragment of residues 32 to 139 has been characterized by the measurement of circular dichroism and by the examination of the ability to bind heme. A preliminary result indicates that the large fragment can bind heme to form a structure similar to that assumed in the intact myoglobin.

Significance to Biomedical Research: It has been shown by the works of several groups of investigators that smaller fragments of myoglobin contain much less helicity than that expected for the "native" structure. The determination of the minimum covalent structure of myoglobin which form the functional structure with heme would give basis to understand the mechanism of folding of myoglobin and the process of evolution of this protein in relation to that of hemoglobin.

Serial No. NIAMD-LCB-4
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Structure-function studies on Staphylococcal nuclease and Nuclease-T.

Previous Serial Number: NIAMD-LCB-3

Principal Investigators: Guillermo R. Sanchez
Irwin M. Chaiken

Other Investigator: Christian B. Anfinsen

Man Years:

Total: 1.4
Professional: 1.4

Project Description:

Objectives: The main objective of this work has been to study which amino acid residues in staphylococcal nuclease-T define the proper structure and function of this enzymic species. Nuclease-T is the active derivative obtained by selective trypsin digestion of staphylococcal nuclease. It consists of two fragments containing residues 6 through 48 and residues 49 through 149 of the native enzyme, respectively, complexed in a noncovalent manner.

A further objective of this work has been to correlate the results with the known three-dimensional structure of nuclease, and thus to implicate certain interactions in the folding of the polypeptide chain into the active conformation.

Methods Employed: The basic method employed has been Merrifield's method of solid phase peptide synthesis of the smaller fragment of nuclease-T. Peptides have been prepared possessing the sequence 6-47, with substitutions of different amino acid residues at specific points along the sequence. In addition, peptides have been prepared with truncations at the N-terminus and at the C-terminus. These peptides were assayed for their capacity to bind native fragment 49-149 and to generate DNase and RNase activity. The results have been compared with predictions based on the three-dimensional model of nuclease.

Major Findings: The substitution analogs have produced evidence that residues isoleucine 18, aspartic acid 19, and tyrosine 27 are important residues in the 6-47 sequence (these observations add to the previous finding that residues aspartic acid 21, arginine 35, aspartic acid 40, and glutamic acid 43,

perform important roles in nuclease-T). It has been found that replacement of isoleucine 18 with leucine brings about essentially total loss of activity, and replacement of aspartic acid 19 with alanine causes complete loss of activity. In both cases the ability of the synthetic peptides to bind native 49-149 remains, although it is somewhat diminished. The need for a nonpolar site at residue 27 has been studied by replacement of tyrosine 27 with phenylalanine, glutamic acid, and leucine. The observations on the alanine 19 analog have been interpreted as meaning that aspartic acid 19 provides a fourth ligand around the essential calcium atom at the active site, in agreement with the crystallographic studies, which show this residue to be sufficiently near the calcium atom for interaction. The effect of substituting isoleucine 18 with leucine may be interpreted as a disruption of the interaction of the β-carboxyl group of aspartic acid 19 and calcium. The importance of tyrosine 27 is in agreement with the crystallographic work, which shows the side chain of this residue in a tightly packed hydrophobic pocket which includes the side chains of residues phenylalanine 34 and 76, among others. In addition, there appears to be an H-bond network between the tyrosyl-27 hydroxyl, the glutamyl-10 γ-carboxyl, and the lysyl-28 ε-amino groups.

The synthesis of fragments with truncations at the N-terminus has shown that loss of residue 9 brings about a large loss of activity and of the ability to bind native 49-149. Loss of residue glutamic acid 10 causes total loss of activity. It has been observed, however, that in the presence of the active site ligands Ca^{++} and deoxythymidine-3',5'-diphosphate total activity is not lost until residue threonine 13 is lost. This has been interpreted as being due to the stabilization of the active complex formed.

The synthesis of fragments with truncations at the C-terminus has shown that residues 45 through 48 are not necessary for the formation of an active complex. Loss of residue threonine 44 brings about total loss of activity, but binding remains excellent. Because a synthetic peptide with the sequence 9-44 but with alanine in place of threonine at position 44 also gives activity when combined with native 49-149, it seems that it is the peptide bond between residues glutamic acid 43 and threonine 44 that plays an important role in the proper orientation of the γcarboxyl group of glutamic acid 43 with Ca^{++} at the active site of nucleaseT. Such a role is played through the neutralization of the α-carboxyl group of glutamic acid 43.

The fact that the synthetic fragment 9-44, Ala 44 produces activity when incubated with native 49-149 indicates that the sequence 9-44 contains sufficient information for the formation and the stabilization of an active complex. This sequence represents a 20% reduction in the native sequence of residues, a significant finding, also to be reflected in the reduced time of synthesis of future analogs.

Significance to Biomedical Research: This project is contributing to the understanding of precise interactions within a protein molecule which stabilize the native structure. It appears that important rules applicable to protein systems in general will evolve during the course of this work.

Proposed Course of Project:

1) Further characterization of the interaction between glutamic acid 43 and Ca^{++} at the active site is intended.

2) Purification of the important semisynthetic complexes will be carried out.

3) It will be of interest to define the transition point, along the C-terminus of the smaller fragment of nuclease-T, at which the ability to form a complex with 49-149 is lost.

4) The role of the β-pleated sheet structure (present in residues 12 through 35 in nuclease) in the formation and stabilization of the native structure will be studied.

5) The use of some of the synthetic peptides prepared in this work (for instance, 6 through 44 and 9 through 44) in a proton magnetic resonance study of the folding of staphylococcal nuclease is also intended.

6) It is hoped that the synthetic methods developed will be of value in the future synthesis of native fragment 49-149 and of nuclease itself.

Publications:

Chaiken, I.M.: Chemical studies of structural features in staphylococcal nuclease-T', J. Biol. Chem., in press.

Serial No. NIAMD-LCB-5
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Preparation and studies of semisynthetic protein complexes.

Previous Serial Number: NIAMD-LCB-4

Principal Investigator: Irwin M. Chaiken

Other Investigators: Guillermo R. Sanchez
John Griffin
Jack S. Cohen

Cooperating Units: 1) Physical Sciences Laboratory, Division of Computer Research and Technology, NIH (Jack S. Cohen: some of this work reported in annual report PSL-DCRT-).
2) Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada (Murray H. Freedman).

Man Years:

Total: 1.2
Professional: 1.2

Project description:

Objectives:
1) To prepare, by chemical synthesis, polypeptides and protein fragments which interact with corresponding native polypeptides or protein fragments to form biologically active complexes.
2) To obtain purified synthetic products suitable for detailed study as part of semisynthetic protein complexes.
3) To use the semisynthetic complexes for studying the conformational and functional properties of the particular systems in question, notably by studies of synthetic analogues.
4) To use such semisynthetic complexes also as models for investigating the application of various new kinds of techniques for studying proteins and interacting protein systems.

Methods Employed: Several systems have been investigated, including staphylococcal nuclease-T', pancreatic ribonuclease S', and oxytocin-neurophysin. In each of the two former cases, a specific protein fragment has been prepared by solid phase peptide synthesis and purified by the functional property of noncovalent complex formation with the complementary native protein

fragment. Thus, synthetic-(6-47), the peptide corresponding to residues 6 through 47 of S. nuclease, has been prepared and combined noncovalently with the complementary native fragment, nuclease-T-(49-149). The resultant semisynthetic complex, semisynthetic nuclease-T', can be isolated in a state almost as active as native nuclease-T' and therein serves as a basis for studying nuclease-T' analogues by incorporating synthetically-derived analogues of synthetic-(6-47). The same approach as that described for semisynthetic nuclease-T' has been used for semisynthetic ribonuclease S', which contains synthetic-(1-15)(the peptide corresponding to residues 1 through 15 of ribonuclease) and native ribonuclease-(21-124).

Most recently, experiments have been undertaken to study the interaction of the peptide hormone oxytocin with neurophysin, the latter of which is a neurohypophyseal oxytocin-binding protein. Again, the approach is to synthesize the nonapeptide oxytocin, and analogues of oxytocin, for which the interactions with neurophysin can be studied by various techniques.

Major Findings: For semisynthetic nuclease-T', experiments have been completed on an active site analogue containing aspartic acid at position 43 in place of the normally occurring glutamic acid. [Asp 43]-Semisynthetic nuclease-T' has an overall conformation similar to that of native nuclease-T' but lacks enzymic activity. Nonetheless, the complex does bind the active site ligands Ca^{++} and deoxythymidine-3',5'-diphosphate, thus defining the critical role of glutamic acid 43 as a direct participant per se in catalysis by nuclease-T', and perhaps for that by intact nuclease as well. The isolation of other semisynthetic nuclease-T' analogues is in progress.

Analogues of semisynthetic ribonuclease S' have been made for active site and conformationally important residues in the (1-15) fragment. In one set of experiments, ^{13}C-enriched and ^{19}F-containing synthetic-(1-15) peptides have been prepared and used to study the interaction of the (1-15) fragments with ribonuclease-(21-124) by carbon 13 and fluorine 19 nuclear magnetic resonance. It has been shown that such selective enrichment can be achieved synthetically and that the products provide valuable tools for measuring structural parameters for specific residues in going from disordered (denatured) to ordered (native) environments. The techniques involved also have indicated the application to the study of the mechanism of action of ribonuclease A and ribonuclease S.

Experiments on oxytocin-neurophysin are in the preliminary stage.

Significance to Biomedical Research: The above studies allow the description of several structural bases for conformation and biological function for the specific proteins in question. Conclusions for the specific cases of study are applicable to an understanding of other proteins as well. In addition, the work being carried out with semisynthetic protein complexes should provide a basis for the understanding and study of other biologically important interacting protein systems.

Proposed Course of Action:

1) Continuance of projects for semisynthetic nuclease-T' and ribonuclease S', with specific attention to important synthetic analogues for which

functional and conformational information will lead to an increased understanding for these proteins specifically and for other proteins in general.

2) Study of the oxytocin-neurophysin system by synthesis of oxytocin but also perhaps by chemical studies of neurophysin.

3) Use of the above systems as models for investigating the applicability of various techniques, including several types of nuclear magnetic resonance spectroscopy for studying interacting protein systems in general.

Serial No. NIAMD-LCB-6
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Kinetic studies of the mechanism of action of staphylococcal nuclease.

Previous Serial Number: None

Principal Investigator: Ben M. Dunn

Other Investigators: Irwin M. Chaiken
Carlo DiBello
Christian B. Anfinsen

Cooperating Units: Heuristics Laboratory, Division of Computer Research and Technology, NIH

Man Years:

Total: 1.5
Professional: 1.5

Project Description:

Objectives:

1) To determine the kinetic parameters (k_{cat} and K_m) for hydrolysis of deoxythymidine 5'-p-nitrophenyl phosphate 3'-phosphate by staphylococcal nuclease as a function of pH in aqueous solution.

2) To determine the analogous parameters in solutions of deuterium oxide (D_2O) to ascertain if a significant solvent isotope effect is associated with this reaction.

3) To determine the position of bond cleavage in the substrate by studying the products formed in $H_2{}^{18}O$ enriched water.

4) To more carefully determine the inhibition patterns and inhibitor constants (K_i) for deoxythymidine 3',5'-diphosphate (pdTp) as a function of pH.

5) To examine in detail the influence of modification of carboxylic acid side chains in the protein, and determine the influence of pH upon this reaction.

Methods Employed: The hydrolysis of the substrate deoxythymidine 5'-p-nitrophenyl phosphate 3'-phosphate has been studied by following release of the product p-nitrophenyl phosphate at 330 nm. Hydrolysis rates were determined over a range of conditions of pH and substrate concentration in the solvents H_2O and D_2O. The parameters k_{cat} and K_m were obtained by curve fitting

to the appropriate function accounting for the substrate inhibition observed at high substrate concentration. The hydrolysis was also carried out in solutions enriched in $H_2{}^{18}O$, the products isolated, and examined for ^{18}O content by combined mass spectroscopy-gas chromatography. Inhibition of the reaction by (pdTp) was studied kinetically over a range of inhibitor concentration at two different substrate concentrations and at several pH values. Equilibrium dialysis was employed to measure the binding constants and stoichiometry.

The enzyme was incubated at controlled pH values with various concentrations of Woodward's reagent K (N-ethyl-5-phenylisoxazolium-3'-sulfonate) and aliquots were withdrawn and assayed for DNase activity at measured time intervals. The rate of inactivation was determined in this fashion, and the variation of this rate with pH was measured. Because the reagent undergoes solvent catalyzed transformations at the pH values utilized, and these reactions affect the observed rate of inactivation, the time course of these reactions was independently determined at pH's in the range under study.

Major Findings: The variation of the kinetic parameters with pH has allowed the determination of the ionization constants of functional groups of the protein involved in substrate binding and catalysis. This data will aid in the further elucidation of the identity of these critical residues. The comparison of the pH independent plateau rates for reactions in H_2O and D_2O has revealed the lack of a significant solvent isotope effect. This result will place restrictions on the mechanistic possibilities and will aid in elucidation of the mode of catalysis. Examination of the incorporation of ^{18}O from solvent water has confirmed the cleavage position as occurring between phosphorous and oxygen. This information will also aid in delineating the mechanism.

Under a variety of conditions, the inhibition by pdTp has yielded complex, non-linear effects, suggesting strongly that multiple binding to the enzyme is occurring. Attempts to quantitate this phenomenon using equilibrium dialysis have met with limited success but the synthesis of inhibitor with higher specific activity will aid in this goal.

Woodwards reagent K, known to react specifically with carboxylic acid groups, provides a rapid and complete inactivation of the enzyme. The reaction has been characterized with respect to rate variation with pH, the influence of added calcium plus pdTp, reagent K concentration, enzyme concentration, and the influence of the degradative hydrolysis of the reagent. The observation of a kinetically significant ionization constant in this process will allow the assignment of this pKa to a functional residue perhaps involved in the maintenance of essential tertiary structure.

Significance to Biomedical Research: Elucidation of the mechanism of nuclease catalysis will provide a deeper understanding of enzyme action in general. Understanding the inhibition of this enzyme by reversible and irreversible inhibitors will allow the more rational design of specific inhibitors directed against this protein.

Proposed Course of Project:

1) Further studies of the stoichiometry and mechanism of inhibition by nucleotides and oligonucleotides by equilibrium dialysis and kinetics.

2) Study of the stoichiometry and specific sites of modification of the nuclease by Woodward's reagent K.

3) Determination of the influence of variation of substrate electronic properties upon the kinetic parameters.

Serial No. NIAMD-LCB-7
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Nuclear magnetic resonance studies of ribonuclease, myoglobin and cytochrome c.

Previous Serial Number: NIAMD-LCB-7

Principal Investigators: Alan N. Schechter
John H. Griffin
David H. Sachs
Waldo Fisher
Hanspaul Hagenmeier
Ann Eastlake

Cooperating Unit: DCRT, LPS (Jack S. Cohen)

Man Years:

Total: 1.6
Professional: 1.4
Others: 0.2

Project Description:

Objectives: Nuclear magnetic resonance techniques give information useful to understanding the structure of proteins in solution and the interactions of proteins with substrates and other ligands. At 220 MHz it is possible to resolve proton signals from the different aromatic residues and make inferences about the electronic environment of these residues in the three-dimensional structure of the protein. These inferences may be tested by studies of suitable chemical model compounds and by computation of the data with various mathmatical models. The development, at the NIH and elsewhere, of methods for studying the resonances of other nuclei (^{13}C, ^{31}P, etc.) and of Fourier transform analysis will further expand the applicability of these methods to the study of macromolecular structure.

Methods Employed: The Varian 220 MHz nuclear magnetic spectrometer with a probe for recording proton resonances has been used for most of our work. Protein samples have been exchanged with pure D_2O and residual protons have been measured and recorded on a Varian time averaging computer. pH titrations have been accomplished by measuring time-averaged spectra at closely spaced pH values. Temperature, ionic strength, and the concentration of substrate analogs have been used as independent variables. Selected chemical modifica-

tions of proteins have also been accomplished for the purpose of identifying certain resonances.

Many spectra have been digitized, recorded on magnetic tape, and then analyzed into their component Lorentzian curves with an IBM 360/50 computer and 2250 display so that accurate values of chemical shift, area, and peak width could be obtained. Theoretical titration curves of chemical shift vs. pH for the individual resonances have been computed for the Henderson-Hasselbalch relationship and for interacting titrating groups. These curves have been compared to our data by computer fitting.

Major Findings: The imidazole C2 protons of the four histidine residues of bovine pancreatic ribonuclease have been resolved from other resonances and titration curves of chemical shift vs. pH constructed. Only one of the four curves fits the Henderson-Hasselbalch titration curve. The other three show acid perturbations and one shows an alkaline perturbation as well. On the basis of previous studies of histidine model compound titration curves and mathematical models for interacting groups, these acid and alkaline effects can be ascribed to the existence of neighboring titrating groups. These groups may be tentatively ascribed to lysine and aspartic acid or glutamic acid residues.

The assignment of these resonances to corresponding histidine residues has been done by reference to work published by others. On this basis, and by comparison to the X-ray model, our results suggest that the active site histidine residues 12 and 119 are interacting with lysine 41 and aspartic acid 121. However, because of the importance of these assignments we have attempted to identify the resonances ourselves, by deuteration of histidine 12 in the S-peptide of ribonuclease S. The results are still not unequivocal but should allow us to uniquely assign these two active site histidine residues.

The effects of substrate analogs, including uridine and cytidine monophosphates and a new class of nucleotide phosphonates on these titration curves have been studied. The mononucleotides give effects which can be interpreted with reference to the base and the position of the phosphate. The dinucleotide phosphonate seems to be a good analogue of the substrate and has allowed us to measure the pK values of the various protons in the active site and in the inhibitor and to make inferences about the geometry of the active site.

Titration curves of chemical shift vs. pH have been constructed for the histidine residue of sperm whale and horse heart metmyoglobin. Comparisons of these have allowed the assignment of one histidine residue to a resonance. The effect of changing the state of ligation of the iron has been detected in the resonance of another histidine. Marked changes in these spectra also occur upon removal of the heme group or fragmentation of the enzyme.

Cytochrome c has been studied in an analogous way and the pK of the one titrating histidine residue has been determined. The effects of various chemical and physical modifications of the protein have been studied.

The new nuclear magnetic resonance spectroscopic instruments at NIH for measuring ^{13}C and ^{31}P by continuous wave and pulsed Fourier transform methods have been applied to several proteins and their substrates with encouraging preliminary results.

Significance to Biomedical Research: These methods allow the study of the behavior of different parts of a protein molecule in solution--thus combining advantages of the usual spectroscopic methods and X-ray crystallography. The results from the imidazole compounds and ribonuclease indicate that the imidazole proton titration curves are sensitive probes to electronic interactions within a small compound or even a protein. The work with substrate analogs is allowing a definition of electronic aspects of their interactions with different parts of the protein molecule. The new work with myoglobin and cytochrome c facilitates the study of the conformation and function of the important class of heme proteins by an analysis of the environments of their histidine residues. The ^{13}C and ^{31}P methods enlarge the number of residues and atomic groups that may be studied. These methods will improve our understanding of the mechanism of action of these, and possibly other, proteins.

Proposed Course of Project: The collection and analysis of NMR data on these and other proteins will continue in an attempt to enlarge our understanding of protein structure-function relations in solution.

Publications:

Sachs, D., Schechter, A.N. and Cohen, J.S.: Nuclear magnetic resonance titration curves of histidine ring protons. I. Influence of neighboring charged groups. J. Biol. Chem. 246, 6576-6580, 1971.

Shrager, R.I., Cohen, J.S., Heller, S.R., Sachs, D.H., and Schechter, A.N.: Mathematical models for interacting groups in nuclear magnetic resonance titration curves. Biochemistry 11, 541-547, 1972.

Serial No. NIAMD-LCB-8
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Immunologic properties of a helical portion of staphylococcal nuclease.

Previous Serial Number: NIAMD-LCB-6

Principal Investigators: David H. Sachs
Alan N. Schechter
Christian B. Anfinsen
Ann Eastlake

Man Years:

Total: 1.8
Professional: 1.0
Other: 0.8

Project Description:

Objectives: The C-terminal cyanogen bromide fragment of staphylococcal nuclease (piece E), a polypeptide of 50 amino acids, binds to another enzymatically inactive fragment of staphylococcal nuclease (Piece 1-126) to generate activity. At least one of the antigenic sites of intact nuclease has been localized to this Piece E fragment. In addition, the crystallographic model of nuclease shows a large α-helical content in this region. On the hypothesis that this region might therefore be involved in the folding of the polypeptide chain, we have set out to prepare antibodies specific for a single antigenic determinant in this region of the nuclease molecule. We wish to use these antibodies to study the conformational equilibria of Piece E and other polypeptide fragments of nuclease in solution.

Methods Employed: Precipitating antisera against native nuclease, Piece E, and Piece 1-126 have been produced in goats. The antisera have been purified by selective immunoabsorption on Sepharose columns to which proteolytic fragments of nuclease had been covalently bound. By this means antibodies to a single antigenic determinant in the region (99-126) have been prepared. The monospecificity of these antibodies has been determined by their behavior during ultracentrifugation in the presence of nuclease. Further characterizations of antibody specificities by Ouchterlony double diffusion in agar, precipitin curves, immunoelectrophoresis and passive hemagglutination have been performed.

Major Results: Because antibodies to a single antigenic determinant in the region (99-126) of nuclease do not precipitate with nuclease but do inactivate it, assays of binding by spectrophotometric means have been possible.

A kinetic analysis of the interaction of these antibodies with nuclease has been performed using the spectrophotometric assay of nuclease activity as a sensitive monitor of unbound nuclease. In addition to providing kinetic and equilibrium parameters of this interaction, this analysis also provides an exquisitely sensitive assay of antibody (in the range of 10^{-8} molar).

The analysis has been extended to the binding of these antibodies to Piece E, using the kinetic analysis to measure residual unbound antibody. The results have been found to be consistent with a model in which the polypeptide fragment exists in solution in a conformational equilibrium between many disordered conformations and a "native format" conformation similar to that which obtains in the native nuclease molecule. Assuming that the antibodies interact effectively only with the native format, a conformational equilibrium constant ($K_{conf.}$) has been determined from the data. The value of $K_{conf.}$ for Piece E is 2×10^{-4}, indicating that approximately 0.02% of the polypeptide fragment in solution is folded in the native format with respect to the antigenic determinant.

Significance to Biomedical Research: There are many naturally occurring proteins which are not easily studied in their intact state, such as membrane proteins, lipoproteins, and multiple-subunit proteins. Since conformation often plays a major role in the biologic properties of such proteins, one needs means of assessing the conformational properties of fragments or subunits of these proteins. Because such fragments and subunits may contain only a very small percentage of the corresponding native conformation, there are often no physical methods capable of such assessment. The immunologic analysis of a K_{conf} therefore provides a parameter of conformation unobtainable by physical methods alone. It may therefore prove valuable in elucidating the structure and function of more complicated protein systems.

Proposed Course of Project: The same analysis will be applied to fragments of different lengths containing the same relevant antigenic determinants. This should give an indication of the importance of cooperative interactions in elevating K_{conf}. Certain changes of amino acid sequence which deter or promote helix formation will be made by use of solid-phase synthesis, and the effects on $K_{conf.}$ of such changes will be assessed.

Serial No. NIAMD-LCB-9
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Improvements in the solid-phase synthesis of polypeptides.

Previous Serial Number: NIAMD-LCB-9

Principal Investigators: David H. Sachs
Lila Corley
Christian B. Anfinsen

Man Years:

Total: 1.4
Professional: 0.4

Project Description:

Objectives: The times usually devoted to the required steps in the Merrifield solid-phase synthetic scheme do not permit the manual addition of more than about two or three amino acid residues per day. This means that considerable time is needed even for the synthesis of rather short polypeptides, as we have found in our previous syntheses of nuclease fragments. We have therefore set out to modify the synthetic scheme in order to increase the speed of synthesis and to minimize those losses attributable to the long reaction times under harsh conditions which are presently used in this method.

Methods Employed: Methods were developed for following the coupling and deblocking reactions during solid-phase synthesis in small funnels so that the time course could be determined. An apparatus was constructed permitting the rapid addition of reagents with mixing by bubbling rather than by rocking. The process of shrinking and swelling the resin in order to increase washing efficiency was studied and employed.

Major Findings: Both the coupling and deblocking reactions were found to proceed much faster than was originally anticipated. In addition, the loss of peptide from the resin was found to be linear with time of exposure to TFA. Therefore it seemed that much faster reaction times would be advantageous. Washing by shrinking and swelling was found to be superior to the standard washing technique.

Using these modifications the solid-phase synthesis of bradykinin, a naturally occurring nonapeptide, was performed in less than five hours. The material was fully active by a biologic assay.

Significance to Biomedical Research: Former methods of solid-phase synthesis required at least four days for the synthesis of bradykinin. Five hours therefore represents a marked saving of time. If this method can be extended to other peptides it should enable investigators to study many more problems by this method than could previously have been studied in a comparable time.

Proposed Course of Project: The method will be applied to other biologically important polypeptides such as fragments of staphylococcal nuclease.

Publications:

Parikh, I., Corley, L., and Anfinsen, C.B.: Semi-synthetic analogues of an enzymically active complex formed between two overlapping fragments of staphylococcal nuclease. J. Biol. Chem. 246: 7392-7397, 1971.

Serial No. NIAMD-LCB-10
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Solid phase synthetic studies on staphylococcal nuclease.

Previous Serial Number: NIAMD-LCB-8

Principal Investigators: David East
Alan N. Schechter
Christian B. Anfinsen

Man Years:

Total: 0.5
Professional: 0.5

Project Description:

Objectives: To assign uniquely the histidine C-2 proton NMR peaks of staphylococcal nuclease.

Methods and Major Findings: Of the four histidine C-2 proton NMR peaks of nuclease only that of His 124 is assigned. Nuclease-T comprising fragments 6-48 and 49,50-149 has enzymic activity, has been crystallized, and from the X-ray diffraction pattern has been shown to be highly isomorphous with nuclease. It is thus probable that the conformations of Nuclease-T in solution include those of nuclease.

Fragment 49-149 also gives active complexes with the 6-47 Gly 46 His 8 and with 9-47.

We have prepared native fragments 6-48, 50-149 and obtained their NMR spectra and titration curves as a function of pH at 22°C. The combination of these fragments has however yielded NMR spectra significantly different from that of nuclease in spite of the addition of calcium and the substrate inhibitor thymidine diphosphate.

The following peptides have been made by the solid phase method (Merrifield, J. Am. Chem. Soc. 85, 2149)

6-47 His 46 Gly 8
6-47 Gly 46 His 8
6-47 Gly 46 Gly 8
6-47 His 46 His 8 (the native sequence)

and have enzymic activity on complementation with 49-149.

When conditions in which nuclease-T gives a nuclease-like NMR spectrum have been found, the combination of the above fragments with native 49-149 will enable us to uniquely assign the C-2 proton resonances to their respective histidines.

Significance to Biomedical Research: The environment of several protons may be probed simultaneously by the nuclear magnetic resonance method. Of the resonances resolved to date perhaps the most important are those of histidine C-2 ring protons since histidine residues are often involved in the catalytic sites of enzymes. It is important to demonstrate methods by which the individual resonances, especially those from catalytic site histidines, may be assigned. These reporter resonances will give detailed information on the mechanism of catalysis not otherwise obtainable.

Proposed Course of Project: The preparation of these fragments by solid phase synthesis and their purification will be continued. The factors which determine the spectral properties of nuclease-T will be investigated in preparation for the assignment of resonances to their corresponding histidine residues.

Publications:

Epstein, H.F., Schechter, A.N., and Cohen, J.S.: The folding of staphylococcal nuclease: magnetic resonance and fluorescence studies of individual residues. Proc. Nat. Acad. Sci. U.S. 68, 2042-2046, 1971.

Serial No. NIAMD-LCB-11
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Nuclear magnetic resonance and fluorescence spectroscopic studies on staphylococcal nuclease.

Previous Serial Number: NIAMD-LCB-7 and 9

Principal Investigators: David East
Alan N. Schechter

Other Investigator: Christian B. Anfinsen

Cooperating Units: Jack S. Cohen (DCRT/PSL)
Evert Nieboer (Laurentian University, Sudbury, Ontario)

Man Years:

Total: 0.8
Professional: 0.8

Project Description:

Objectives: To study conformational transitions in staphylococcal nuclease, to investigate the effects of ligands on the histidine residues of nuclease, to determine whether or not conformational changes are induced by the binding of substrate and to obtain the geometry of the active site in solution.

Methods and Major Findings: The acid-induced conformational change in staphylococcal nuclease has been measured by stopped flowed spectrophotofluorometry of the single tryptophanyl residue. The kinetics of the structural change have been studied as a function of temperature, pH, and ionic strength. Equilibrium aspects of this transition have also been measured by viscosity, circular dichroism, and NMR methods. The details of the earlier findings have been summarized in the previous Individual Project Report (NIAMD-LCB-5) and have been published in the papers listed below. The detailed analysis of the mechanism of the folding of this protein is continuing through the collection and evaluation of further data.

The four histidine C2 ring proton resonances have been resolved at 220 MHz and titration curves of chemical shift values as a function of pH determined. Computer curve fitting has been used to calculate apparent pK values.

The effects of calcium in the range 0.0015 molar to 0.5 molar are two:

i) an increase in pK of all four resonances due to increased ionic strength

ii) a decrease in pK due to the binding of calcium to nuclease and reported solely by resonance H-2 (presumptively Histidine 46).

Thymidine diphosphate, a competitive inhibitor in the presence of calcium ions, increases the tightness of binding for calcium but without evidence of significant conformational change in the enzyme.

The paramagnetic ion Eu^{+++} causes a loss of resonance H-2 and a change in the position of the other three resonances which cannot be explained by the bulk susceptibility effect and which is complete at 1:1 stoichiometry. The physical basis of this affect is being analyzed.

The effects of the diamagnetic ion La^{+++} have also been noted. Broadening of H-2 occurs at pH 5.5, but the basis of this phenomenon is not yet understood.

Significance to Biomedical Research: Whereas other spectroscopic methods yield information about only one site (or the 'average' of many sites) in a macromolecule, the nuclear magnetic resonance method can simultaneously probe several local chemical environments. Further, the use of paramagnetic ions as resonance broadening and shift reagents enables one to calculate distances from the ion-binding site to the reporter protons. This powerful aspect of nuclear magnetic resonance already demonstrated in our studies, may be of importance for the understanding both of physiological and mutant enzymes in solution.

The precise relationship between the genetically determined primary sequence and the active structure of a protein remains one of the major unsolved links in the understanding of protein biosynthesis. Such knowledge may ultimately provide us with the ability to engineer new proteins as well as to clarify how mutant proteins cause human disease. The studies of the dynamic and equilibrium aspects of protein folding and structure will contribute to the knowledge of these relationships.

Proposed Course of Project: We intend to quantitate more accurately the effect of bulk ionic strength on pK with non-bound ions (e.g. Na^{+}, K^{+}, Mg^{++}), to investigate competition for the binding site between Ca^{++} and paramagnetic lanthanides, and to use the latter to determine the distances to the histidine residues. We shall perform further titrations in the presence of thymidine diphosphate and other substrate inhibitiors using the signal of H-2 as a probe of conformational change near the binding site. A mathematical analysis of the kinetic data is also continuing in order to describe the mechanism of folding of this protein.

Publications:

Epstein, H.F., Schechter, A.N., Chen, R.F., and Anfinsen, C.B.: The folding of staphylococcal nuclease: kinetic studies of two processes in acid renaturation. J. Mol. Biol.660: 499-508, 1971.

Anfinsen, C.B., Schechter, A.N., and Taniuchi, H.: Some aspects of the structure of staphylococcal nuclease: Part II. Studies in solution. Cold Spring Harbor Symp. Quant. Biol. 36: 243-255, 1971.

Schechter, A.N.: Chemical and physical factors involved in protein folding as exemplified by staphylococcal nuclease. Part II. Kinetics. PAABS Revista, in press.

Serial No. NIAMD-LCB-12
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Purification and properties of B. megaterium, KM β-galactosidase

Previous Serial Number: NIAMD-LCB-16

Principal Investigator: Harvey Pollard

Other Investigator: Edward Steers, Jr.

Man Years:

Total: 0.2
Professional: 0.2

Project Description:

Objectives:
1) To study the physical, chemical and biologic properties of β-galactosidase from Bacillus megaterium, KM.
2) To investigate influence of subunit association on reactivity of the enzyme.

Methods: The enzyme β-galactosidase from Bacillus megaterium, strain KM, has been purified by affinity chromatography on a medium consisting of (Sepharose-4B)-NH-C_3H_6-NH-C_3H_6-NH-CO-CH_2-CH_2-CO-pNH_2-phenylthio-B-D-galactoside. Molecularly homogeneous monomer and dimer could be prepared on sucrose gradients. The activity of the monomer was studied by both ure denaturation experiments and by immobilization of the monomer on Sepharose 4B. Activity was reconstituted by adding free monomer in 8 M urea, to the Sepharose bound monomer, followed by removal of the urea by dialysis.

Major Findings: The β-galactosidase from B. megaterium has been purified to a state of apparent homogeneity by the technique of affinity chromatograph. This method resulted in 39% of the available activity being recovered in purified form. The entire purification took less than one working day to complete. The main problem in this system was that of understanding the nature of the active species. Studies of the enzyme in solution were ambiguous in terms of whether the monomer was active or not. In the case of B. megaterium β-galactosidase, the spatially isolated monomers were found to be completely inactive. A partial reconstitution of activity was obtained by the addition of free monomer to Sepharose monomer. We interpret these data to mean that the active state of the enzyme in vivo appears to be dimer. This means that the conformation of the active site is in some linked to the joining of subunit binding

sites on the monomers. In addition, free monomers from E. coli β-galactosidase were found to form active hybrids with Sepharose bound B. megaterium β-galactosidase monomers. We conclude from these studies that the free monomer is inactive, and that the dimer is the active species in contrast to E. coli β-galactosidase where only the tetramer form is active.

Significance to Biomedical Research:

1) This system is homologous with the E. coli system and provides a simpler approach to the problem of subunit-subunit interaction.

2) The clear dependence of activity upon association to the dimer may provide insight into the significance of multisubunit enzymes.

Proposed Course of Project: Terminated

Serial No. NIAMD-LCB-13
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Isolation and characterization of trichocysts from Paramecium aurelia.

Previous Serial Number: NIAMD-LCB-17

Principal Investigator: Sidney Pollack

Other Investigator: Edward Steers, Jr.

Man Years:

Total: 1.8
Professional: 1.8

Project Description:

Objectives: To isolate and characterize the structural proteins from trichocysts of Paramecium aurelia; to compare the proteins found in wild type with those found in various mutant strains; to gain an understanding of some of the processes associated with the development of this cellular organelle.

Methods Employed: Paramecia were grown in five gallon carboys (Steers, E., Jr., PNAS 48: 867, 1962) in culture medium made from grasses. Trichocysts were obtained by differential centrifugation of partially concentrated animals and were thermally solubilized. Polyacrylamide gel electrophoresis and column chromatography were performed on both heat solubilized trichocysts and on acid or base solubilized trichocysts.

Major Findings: The solubilization of trichocysts is salt dependent, as they are most readily solubilized by heating in the absence of any salts. The results from heating trichocyst preparations under various conditions are summarized in the table below:

PERCENT PROTEIN SOLUBILIZED

	Solubilization Media			
	Water	0.5M Sodium Phosphate, pH 7.1, PLUS:		
		No KCl	0.1M KCl	0.6M KCL
Temperature				
40°	0%	0%	0%	0%
50°	0.8	0.8	1.3	1.1

60°	4.6	5.2	5.7	4.8
70°	67.6	14.3	12.3	11.2
80°	7.7	1.6	2.5	1.8
Pellet	19.2	78.1	78.1	81.1
Total	99.9	100.0	99.9	100.0

The pellet represents insoluble material which in the water solubilization experiments consists largely of bacterial contaminants.

Although very little trichocyst material goes into solution either in the presence of salt or at the lower temperatures in the water experiment, the supernatants have an absorbancy maximum near 260 mμ. Tests for DNA were negative, while those for RNA were positive (sugar tests). However, the material was resistant to both RNase and Staphylococcoccal nuclease digestion, and was also dialyzable. This suggests a ribonucleotide may be present.

Polyacrylamide gel electrophoresis of heat solubilized trichocysts yields five bands. On SDS gels only two bands are found, corresponding to molecular weights of 17,000 and 35,000. This latter result is comparable to that of trichocysts solubilized directly in SDS and run on SDS gels.

At the present time, column chromatography and ultracentrifugation studies have produced data in which the former technique suggests a highly aggregative system, while the latter technique does not. Further work is needed to clarify these and other observations before a more definitive characterization of tichocyst proteins is to be reported. Once this has been accomplished, an analysis of various mutants will be undertaken.

Significance to Biomedical Research: This work represents a continuation of our objectives to characterize structural proteins related to the cell surface and cell surface properties using a specialized system in microorganisms. The analysis of genetic mutants will hopefully be useful in elucidating some of the problems of protein assembly into a cellular organelle.

Proposed Course of Project: Following an analysis of wild type and mutant structural proteins of trichocysts, we hope to obtain some information on the types of genetic lesions found in the mutants and thus to find out more about how this organelle, the trichocyst, develops.

Serial No. NIAMD-LCB-14
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Structure of the low density lipoprotein particle from human serum.

Previous Serial Number: NIAMD-LCB-14

Principal Investigator: Harvey Pollard

Man Years:

Total: 0.2
Professional: 0.2

Project Description:

Objectives:
1) Develop a quantitative description of the quaternary structure of low density lipoprotein (LDL).
2) Determine the chemical and physical basis for lipid-protein-interactions in LDL.

Methods Employed: LDL was prepared from human plasma by flotation, and fluorescence and circular dichroism measurements under various conditions were performed. Lipids were determined by quantitative assays of cholesterol and phospholipids, and thin layer chromatography was employed on a qualitative basis.

Major Findings: Electron microscopic images of LDL were scanned with an Isodensitracer and quantitative optical density maps were obtained. The spatial distribution of protein subunits in native LDL was found to be quantitatively distributed on the vertices of a 200 Å dodecahedron. A three-dimensional map of the twenty subunits delineated a central cavity where the neutral lipids apparently reside in native LDL. Removal of all neutral lipids resulted in a disordered quaternary structure.

Proposed Course of Project: Terminated.

Serial No. NIAMD-LCB-15
1. Lab. of Chemical Biology
2. Biosynthesis and Control
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Interaction between the first enzyme for histidine biosynthesis and His-tRNA.

Previous Serial Number: NIAMD-LCB-15

Principal Investigators: John S. Kovach
Tikva Vogel
Robert F. Goldberger
Roger Deeley

Man Years:

Total: 3
Professional: 2
Other: 1

Project Description:

Objectives: On the basis of our previous work on the kinetics of derepression of the histidine enzymes we postulated that the first enzyme for histidine biosynthesis plays a previously unrecognized role in repression of the histidine system in Salmonella typhimurium. Because it has been known for some time that His-t-RNA is required for repression of the histidine system, it appeared to us that one test of our hypothesis is to determine whether the first enzyme for histidine biosynthesis interacts with His-tRNA. Using crude tRNA, we previously found that the enzyme had a high affinity for His-tRNA and that it bound His-tRNA in preference to tRNA which had been aminoacylated with any other amino acid. In the present study, we used various partially purified species of tRNA, to test the question of whether the enzyme binds the aminoacylated form of His-tRNA in preference to the deacylated form.

Methods Employed: Partially purified preparations of various species of tRNA were obtained from Dr. Donald Kelmers of the Oak Ridge National Laboratory. In addition, $tRNA^{His}$ was purified to homogeneity from Salmonella typhimurium (both wild type and T-mutant) by the published procedure. The G enzyme and histidyl-tRNA synthetase were purified by the published procedures. Binding of the G enzyme to tRNA was studied by using the nitrocellulose filter binding technique of Yarus and Berg.

Major Findings: We found that for species of tRNA other than $tRNA^{His}$, the G enzyme does not distinguish between the aminoacylated and deacylated forms.

In contrast, the G enzyme binds aminoacylated histidyl-tRNA in preference to deacylated $tRNA^{His}$. Thus, the enzyme not only binds the histidine species of tRNA in preference to all other species, but also binds the aminoacylated form of histidine tRNA preferentially. The possibility that histidyl-tRNA carries out its regulatory role as a complex with the G enzyme is consistent with these findings.

Significance to Biomedical Research: The finding that His-tRNA, a macromolecule known to be required for repression of the histidine system, binds with a very high affinity to the first enzyme of the histidine biosynthetic pathway, may be an important clue to understanding the mechanisms which regulate biosynthetic metabolic pathways.

Proposed Course of Project: We plan to study the relative affinities of the G enzyme to wild type His-tRNA and T-mutant His-tRNA to determine whether the regulatory defect in T mutants involves a defect in the ability of the altered His-tRNA to bind to the G enzyme.

Serial No. NIAMD-LCB-16
1. Lab. of Chemical Biology
2. Biosynthesis and Control
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Mutations in the first structural gene of the histidine operon which alter regulation of the operon.

Previous Serial Number: NIAMD-LCB-20

Principal Investigator: John S. Kovach

Man Years:

Total: 1.6
Professional: 0.8
Other: 0.8

Project Description:

Objectives: On the basis of previous work done on the kinetics of repression of the histidine operon in Salmonella typhimurium, we suggested that the first enzyme for histidine biosynthesis plays a previously unrecognized role in control of the histidine operon. A prediction of this hypothesis is that certain mutations in the first structural gene (G gene) of the operon would lead to alterations in the structure of the first enzyme which affect regulation of the histidine operon. Although a number of mutations affecting the catalytic function of this enzyme are known, none of these affect the process of repression. We have been attempting to find mutations in the first structural gene which do alter the repression process.

Methods Employed: Several selection procedures have been used. As reported previously, initial attempts to obtain mutations in the G gene involved selecting histidine auxotrophs from a strain containing two normally functioning genes for each of the histidine biosynthetic enzymes except for the G gene which is represented only once. Theoretically, all histidine auxotrophs derived from this strain should have mutations in the G gene. Although this method has led to the isolation of several strains bearing mutations in the G gene, this has been an extremely rare event, and none of these auxotrophs shows any change in the process of repression.

To be certain that every organism selected for study contains a new mutation in the G gene another procedure was tried. A large number of prototrophic revertants of several different complete G mutants were examined for alterations in regulation of the histidine operon. None was found to have a regulatory defect.

A third approach has been the selection of feedback resistant mutants, mutants in which the G enzyme is catalytically active but insensitive to inhibition by histidine. We had previously demonstrated that such mutants are

altered in their ability to be repressed. To further study this phenomenon, we developed a procedure which allowed the direct selection of feedback resistant mutation in the G gene of episomes carrying the histidine operon. Twenty feedback resistant episomes were isolated. Several were found to produce a G enzyme highly resistant to inhibition by histidine. Using these episomes to make merodiploid organisms, we were able to demonstrate that the effect of the G enzyme on repression of the histidine operon was trans dominant (see Project Report #17). An exciting finding of these studies was that one of the feedback resistant episomes is not under repression control. This strain may be the first representative of the class of constitutive G gene mutations predicted by our hypothesis.

Major Findings: An episome bearing the histidine operon containing a mutation to feedback resistance has been isolated. The strain carrying this episome is not under repression control. This finding supports the hypothesis that the G enzyme plays a direct role in repression of the histidine operon.

Significance to Biomedical Research: If alterations in the first enzyme for histidine biosynthesis which affect repression of the histidine operon are found, they will constitute proof of our hypothesis, that a biosynthetic enzyme may have regulatory properties which allow it to control its own synthesis as well as the synthesis of the other biosynthetic enzymes.

Proposed Course of Project: Terminate after publishing results.

Serial No. NIAMD-LCB-17
1. Lab. of Chemical Biology
2. Biosynthesis and Control
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The nature of the effect of alterations in the first structural gene of the histidine operon on repression of the operon.

Previous Serial Number: NIAMD-LCB-16

Principal Investigators: John S. Kovach
Tikva Vogel

Other Investigator: Robert F. Goldberger

Man Years:

Total: 0.5
Professional: 0.2
Other: 0.3

Project Description:

Objectives: On the basis of previous studies on the kinetics of repression of the histidine operon in Salmonella typhimurium, we suggested that the first enzyme for histidine biosynthesis (G enzyme) plays a previously unrecognized role in control of the operon. Subsequent studies showed that organisms producing a feedback resistant G enzyme could not be repressed by triazolalanine (TRA), an analogue of histidine which does repress organisms producing wild type G enzyme. We wanted to determine whether this effect of the G enzyme on repression is cis or trans, dominant or recessive.

Methods Employed: An episome bearing a wild type G gene was introduced into a feedback resistant strain. The merodiploid was tested for its ability to be repressed by TRA. Similarly, an episome bearing a mutation in the G gene which renders the G enzyme feedback resistant was introduced into a strain with a normal G gene, and this merodiploid was tested for its ability to be repressed by TRA.

Major Findings: Whereas the feedback resistant strain itself was not repressible by TRA, the merodiploid of this strain containing a wild-type episome was repressible by TRA. Whereas the feedback resistant episome itself (in a deletion strain) was not repressible by TRA, this episome in the strain containing a wild-type G gene was repressible by TRA. These findings demonstrate that the effect of the normal G enzyme acts in a trans dominant fashion. We conclude that the enzyme exerts its effect on the repression process not by acting locally at its site of synthesis but by acting as a freely diffusible

gene product, as expected for a regulatory molecule such as a repressor.

Significance to Biomedical Research: The fact that the first enzyme of a biosynthetic pathway has a regulatory effect on its own biosynthesis and on the synthesis of those enzymes encoded in the same operon may be important to the elucidation of the mechanisms controlling biosynthetic pathways.

Proposed Course of Project: To be terminated after results are published.

Serial No. NIAMD-LCB-18
1. Lab. of Chemical Biology
2. Biosynthesis and Control
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Interaction between the first enzyme for histidine biosynthesis and Ø80dhis DNA.

Previous Serial Number: None

Principal Investigators: Tikva Vogel
John S. Kovach
Robert F. Goldberger

Other Investigators: Mark Levinthal
Carmello Bruni
Francesco Blasi

Man Years:

Total: 1.5
Professional: 1.0
Other: 0.5

Project Description:

Objectives: We previously proposed that the first enzyme for histidine biosynthesis, the G enzyme, plays a role in regulation of the histidine operon. If this enzyme acts at the genetic level, it would be expected to interact with some regulatory element in the DNA of the histidine operon. The recent isolation of a phage carrying the histidine operon in place of some of its own genes has made it possible to test this possibility.

Methods Employed: Radioactively labelled and unlabelled DNA was isolated from Ø80 and from Ø80dhis. Interaction between these preparations of DNA and the G enzyme was studied by the filter binding assay used by Riggs and Bourgois to study binding of the lac repressor to the lactose operon. Competition between cold Ø80dhis DNA and radioactive Ø80dhis DNA and between cold Ø80 DNA and radioactive Ø80dhis DNA was studied to determine whether there is any specific interaction between the enzyme and the bacterial genes carried in the phage genome.

Major Findings: The enzyme binds Ø80dhis DNA. As expected, this binding is completely inhibited by unlabelled Ø80dhis DNA. However, unlabelled Ø80 DNA was not able to inhibit completely. Approximately 30% of the binding to Ø80dhis DNA is maintained even in the presence of a two thousand-fold excess of unlabelled Ø80 DNA. Thus, the enzyme binds specifically to the bacterial DNA carried in the genome of Ø80dhis. We suggest that it binds specifically to some regulatory element in the histidine operon.

Significance to Biomedical Research: The finding that an enzyme may serve as a regulatory protein controlling the rate of its own biosynthesis may be of general importance in repression control.

Proposed Course of Project: We will study what effect on the binding of G enzyme to Ø80dhis DNA is obtained by the addition of aminoacylated histidine tRNA and deacylated $tRNA^{His}$. We will also study whether the enzyme binds to the DNA of a Ø80dhis in which there is an operator mutation in the histidine operon.

Serial No. NIAMD-LCB-19
1. Lab. of Chemical Biology
2. Biosynthesis and Control
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Regulation of enzyme synthesis in mammalian cells in tissue culture.

Previous Serial Number: None

Principal Investigator: Loren G. Lipson

Man Years:

Total: 1.5
Professional: 1.5

Project Description:

Objectives: To study repression of amino acid biosynthetic pathways in mammalian cells in tissue culture.

Methods: In order to achieve the above objective, a tissue culture laboratory was constructed and equipped. The laboratory was set up so that tissues could be cultured either in culture dishes or in suspension. Six different mammalian liver cell lines were then screened for presence of several amino acid biosynthetic pathways using modified assay systems. In addition several cell lines were adapted to growth in suspension culture in order to increase the cellular yield for enzyme isolations.

Major Findings: Several cellular systems involving the metabolism of amino acids have been found suitable for further study.

Proposed Course of Project: To use several of the above systems to study variation in enzyme level and activity with changes in nutrition, substrates, and growth conditions and to determine the mechanisms of these variations.

I. *PROTEIN STRUCTURE AND MECHANISM OF ENZYME ACTION*

Tryptophan Synthetase

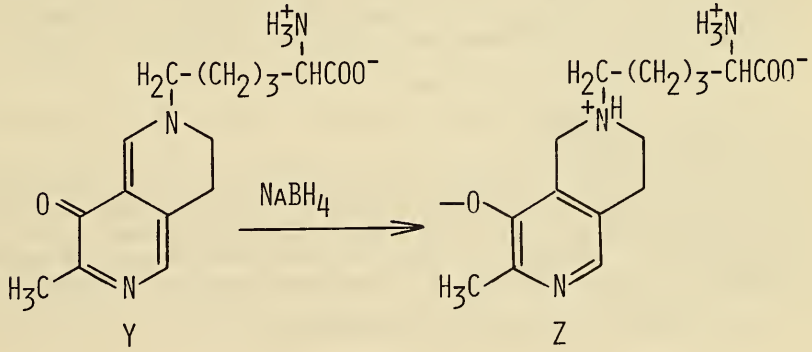
Tryptophan synthetase, an enzyme complex of two different proteins, A and B, is being studied in order to learn more about interaction and control in multienzyme systems. The B protein contains a cofactor, pyridoxal phosphate, which is being used as an optical and chemical probe in studies of the mechanism of the active site.

Studies on chemical modification of histidyl residues of the B protein with diethylpyrocarbonate have shown that one or two histidyl residues are essential for catalytic activity and may serve in abstraction of the α-hydrogen of the substrate. Modified B protein forms nearly normal complexes with its cofactor (pyridoxal phosphate), its substrate (L-serine), and with the A protein.

(Drs. H. Kumagai and E. W. Miles)

α^5-Pyridoxal methylchloride reacts irreversibly at the pyridoxal phosphate-binding site of the B protein. The product of this reaction has been isolated from acid hydrolysates of the protein and shown to be identical to a compound Y synthesized from α^5-pyridoxal methylchloride and N-α-acetyl-L-lysine. The structures of compound Y and its sodium borohydride reduction product Z have been identified by chemical ionization mass spectrometry and by absorbance and nuclear magnetic resonance spectroscopy. These cyclic imine compounds and the reactions leading to their formation have not been previously studied. They are useful models for the study of the optical properties of pyridoxal-amino acid Schiff bases. The enzyme-bound derivative provides a new optical probe for the active site of pyridoxal phosphate-dependent enzymes.

(Dr. E. W. Miles)



Histidine Ammonia-Lyase

Active Site Structure -- Mechanism of Action. Histidine ammonia-lyase, a tetrameric protein, requires divalent metal ions for optimum activity. The affinity of the enzyme for metal ions was shown to be related to the presence of a free cysteinyl residue in each subunit and a single tryptic peptide containing this residue has been isolated and characterized.

(Drs. C. B. Klee and J. A. Gladner)

Previous work from this laboratory showed that the enzyme also contains a dehydroalanine at its active site (R. B. Wickner). I have now shown that incubation of the enzyme with L-cysteine and oxygen results in an irreversible inhibition associated with the incorporation of 3-3.5 moles of cysteine per mole of enzyme. If radioactive cysteine is used, the radioactive material, after acid hydrolysis, is eluted as a single peak different from 1/2 cystine or lanthionine from the amino acid analyzer. I am now trying to identify this derivative and to isolate a peptide containing this residue in order to clarify the nature of the active site of this enzyme and perhaps other related proteins as well. This irreversible inhibition seems to be an active site specific modification since it requires L-cysteine, a competitive inhibitor of the enzyme, whereas D-cysteine or cysteamine are inactive and are also not efficient inhibitors.

(Dr. C. B. Klee)

Lactose Synthetase

Studies with lactose synthetase have concentrated on clarifying the properties of the membrane bound form of the A protein which is apparently very widely distributed among animal tissues. We have found that, in rat brain, the enzyme is found primarily on the synaptosomal (nerve ending) fraction and that it may readily be solubilized from this source with detergents and purified by affinity chromatography on columns of α lactalbumin bound to Sepharose. This partially purified protein is indistinguishable in its kinetic properties from the A protein which we have obtained from bovine milk in pure form. Further studies will be carried out with this system which may help to elucidate the role of this enzyme in membrane synthesis or function.

(Drs. W. Klee and C. B. Klee)

Glycyl tRNA Synthetase

A study of the activation and regulation of glycycl tRNA synthetase, an enzyme with a role in protein synthesis, is being continued. Evidence for an activator substance has been extended and a practical test for its assay further perfected. Separation of the substance from the major UV absorbing and ninhydrin reactive compounds of cell extracts has been achieved.

(Dr. Simon Black and Mrs. Blondel Hazel)

Relationships of Thyrotropin and Exophthalmogenic Factor

Previous work demonstrated that TSH was exophthalmogenic but that partial pepsin digestion of TSH could yield an exophthalmogenic fragment of the TSH molecule which had lost its thyroid stimulating ability. The structure of this fragment has now been carefully characterized; it consists of all of the β subunit and an amino terminal fragment of the α subunit of TSH. This fragment and TSH cause exophthalmos in fish, guinea pigs, and mice. The exophthalmos is associated with an increased synthesis and accumulation of glycosaminoglycans of which hyaluronic acid is the major changing component. Sulfation of glycosaminoglycans parallels the changes in hyaluronic acid and is preceded by SO_4 transport and intracellular activation to PAPS (phosphoadenosine phosphosulfate). TSH and the exophthalmogenic fragment bind to the plasma membranes of guinea pig retroorbital tissue and the binding is associated with adenylate cyclase activation. Gamma globulin from patients with exophthalmos significantly affect binding; this gamma globulin is not LATS (the long acting thyroid stimulating gamma globulin). A mechanism for human exophthalmos has been proposed as a consequence of these data. Experiments designed to test this hypothesis have been initiated.

(Drs. L. D. Kohn and R. J. Winand)

Procollagen and Procollagen Peptidase

Cattle with a recessive anomoly, dermatosparaxis, have been studied. These animals with dramatic skin changes have been shown to accumulate precursor forms of the collagen α_1 and α_2 chains. These precursor forms are larger than the normal α_1 and α_2 chains, the increased size being caused by a peptide extension at their amino termini. Dermatosparaxis has been shown to be caused by a missing enzyme, procollagen peptidase, which cleaves pro α_1 and pro α_2 to yield α_1 and α_2, respectively. The enzyme has been characterized in normal cattle tissues and shown to be elevated in activity in several human disease states. A relationship of procollagen and procollagen peptidase to aging has been postulated. The procollagen chains have been synthesized with an *in vitro* polysome system in a 2 : 1 ratio, *i.e.*, their precursor nature has been definitively established. This system is stimulated by initiation factors.

(Drs. L. D. Kohn, C. M. Lapiere, and A. Lenaers)
(Drs. L. D. Kohn, C. M. Lapiere, S. Kerwar, and H. Weissbach)
(Drs. L. D. Kohn, C. M. Lapiere, P. Bayer, and G. Martin)

Structure-Function Studies of Hydroxypyruvate Reductases

A hydroxypyruvate reductase has been induced in *P. acidovorans* by growth on glyoxylate. After purification to homogeneity, as shown by ultracentrifugation and disc gel electrophoresis, the enzyme is stable in 0.04 M Tris Cl, pH 7.4, containing 25% glycerol and 0.1 M NaCl; it has an $s^0_{25,w}$ of 4.5 S to 5.0 S and a molecular weight of approximately 80,000. The constitutive enzyme, also present in the glyoxylate-grown organism, has values of 4.8 S and 75,000, respectively [*J. Biol. Chem.*, 243, 2492 (1968)]. From SDS gels a subunit molecular weight of 40,000 has been calculated for the induced enzyme. The

induced enzyme has a different amino acid composition, absorption spectrum, and isoelectric point. It has a higher pH optimum, is competitively inhibited by glyoxylate, and has an inversed affinity for reduced pyridine nucleotides, *i.e.*, it has a higher "K_m" for DPNH than TPNH. Both enzymes are specific for hydroxypyruvate; however, in the induced enzyme, the reaction is more readily reversed by DPNH and D(-)-glycerate. The induced enzyme is inhibited by SO_4, Cl, and NO_3; PO_4 exhibits activation and then inhibition, *i.e.*, negative cooperativity. The constitutive enzyme is only activated by these anions. The subunits of the induced enzyme are identical and can reassociate to yield a protein having the same physical and kinetic properties as native enzyme. The yield of reassociation is nearly 100%, the half-time is less than 2 minutes, and the kinetics are compatible with a simple bimolecular reaction. Preliminary evidence indicates that the induced enzyme is a membrane enzyme, whereas the constitutive is cytoplasmic in location.

The amino acid sequence of an active site peptide from glyoxylate reductase has been determined. A striking similarity exists with the sequence of an analogous peptide in glyceraldehyde 3-phosphate dehydrogenase.

(Drs. L. D. Kohn and J. M. Utting)

II. *COMPLEX CARBOHYDRATE*

Malignant transformation of cells is accompanied by alterations in their surface sugars which can lead to alterations in serologic properties. For example, viral-transformed 3T3 cells (mouse fibroblasts) have 50% less sialic acid than normal 3T3 cells. It was not known whether the observed reduction in sialic acid was due to loss of sialic acid in a particular linkage or to a general loss of sialic acid in all linkages. Sialyl linkages are more resistant to acetolysis than are hexosyl or *N*-acetylhexosaminyl linkages and disaccharides containing sialic acid linked to hexoses or *N*-acetylhexosamine can be isolated from the acetolysate. Cells were grown in [^{14}C]glucosamine in order to label their sialic acid. After extraction with TCA, the cell residues were acetolyzed. Disaccharides containing sialic acid were isolated using ion-exchange columns and paper chromatography and their ^{14}C activity determined. With 3T3 mouse cells approximately equal, ^{14}C activity was found associated with the standard disaccharides, NANA-α-2-3-Gal and NANA-α-2-6-Gal; no activity was associated with sailyl disaccharides containing *N*-acetylhexosamine. No change in ratio was found using 3T3 cells transformed with SV40 or MSV viruses. Therefore, the decrease in sialic acid after transformation reported by others [*cf.*, Ohta *et al.*, *Biochim. Biophys. Acta*, 158, 98 (1968) and Wu *et al.*, *Biochemistry*, 9, 5083 (1970)] results from a proportional decrease in both types of linkages.

(Drs. V. Ginsburg, C. A. Hickey, and A. Kobata)

Structure and Assembly of *E. coli* Membranes

The surface of *Escherichia coli* consists of an inner, or cytoplasmic membrane, a rigid cell wall, and an outer membrane. Recent work in another laboratory has indicated that lipopolysaccharide (LPS) is synthesized in the cytoplasmic membrane and then transferred to the outer membrane. We wanted to determine whether such newly completed LPS molecules enter into discrete specialized regions of the outer membrane or whether they are inserted randomly throughout this membrane. We used a mutant of *E. coli* which forms LPS rich in carbohydrate when grown with galactose but forms LPS with half as much carbohydrate when grown without galactose. Since the density of the outer membrane increases with increasing carbohydrate content, fragments of outer membrane from cells grown in the presence of galactose are denser than fragments of outer membrane from cells grown in its absence. These two types of membrane fragments can be separated by equilibrium centrifugation. Cells were therefore grown in the absence of galactose and galactose then added. At various times thereafter membranes were prepared, fragmented by sonication, and centrifuged to equilibrium on sucrose. Within 4 minutes of exposure to galactose, equivalent to 10% of a doubling time, a small percent of the outer membrane fragments were found to have the very high density characteristic of fragments from galactose-grown cells. This result is consistent with the hypothesis that newly synthesized LPS molecules enter the outer membrane in discrete, specialized regions. The results are incompatible with entry randomly throughout the existing membrane. The specialized regions of LPS entry are being isolated, in order to determine their size, composition, and the number per cell.

(Drs. C. F. Kulpa and L. Leive)

Mutants requiring diaminopimelic acid, a constituent of cell wall, or requiring unsaturated fatty acids, needed for surface lipids, are being isolated from the *E. coli* mutant used in the above studies. Diaminopimelic acid auxotrophs will be used to specifically label wall and determine the relationship between cell wall and outer membrane. Fatty acid auxotrophs will be used to determine the effect of altering fatty acid composition on the synthesis, structure, and function of the outer membrane.

(Drs. C. F. Kulpa and L. Leive)

Although the overall composition and biosynthesis of lipopolysaccharides from different coliform bacteria is well known, there has been little information available on whether the LPS of a given organism consists of a homogeneous population of molecules or whether there are fractions of differing composition. The LPS of one strain of *E. coli*, O111:B4, has been isolated by two methods: the classical phenol extraction method and a new aqueous butanol procedure devised in this laboratory. Material isolated by both methods shows two separable fractions that have distinct composition, and distinct physical characteristics as indicated by equilibrium centrifugation, sedimentation, diffusion, and viscosity. One fraction is a long rigid molecule with a very high proportion of antigenic carbohydrate side chains. The other fraction is

more nearly globular, shows a tendency to aggregate, and has a lower proportion of antigenic side chains.

(Drs. D. C. Morrison and L. Leive)

Membrane Function

Bacterial Chemotaxis. Studies have been initiated to determine force-response relationships pertaining to chemotactic motions of *E. coli* K_{12} bacteria. Assay techniques for bacterial motion utilize newly developed methods of laser light intensity correlation spectroscopy. The isolation of chemotacti receptors is also being attempted, with subsequent analysis of receptor function when embedded in reconstituted lipid bilayer membranes.

(Drs. L. D. Kohn and R. J. Nossal)

Membrane Transport. D-Lactate has been demonstrated to stimulate bacterial transport of amino acids and sugars. This enzyme has been partially purified from *E. coli* and shown to be nonsensitive to PCMB, NEM, and other SH inhibitors, whereas these same reagents significantly inhibit bacterial transport. The enzyme can exist in large molecular weight complexes with cytochrome b_3. It contains bound flavin in a form important to enzyme function and is not DPNH or DPN dependent.

(Drs. L. D. Kohn and R. Kaback)

Thyrotropin Activation of the Thyroid. Thyroid membranes have been prepared and characterized. Specific binding of TSH and its subunits has been demonstrated. A new binding assay has been developed which shows that binding is immediate and precedes adenylate cyclase activation.

(Drs. L. D. Kohn and S. M. Amir)

IV. *POLYAMINES*

With Dr. F. Irreverre of this Institute, we have developed a sensitive, automated method for the analysis of amines and amine derivatives with an amino acid analyzer. This is the first method available which is both sensitive and sufficiently specific to permit complete separation of closely related amines. We have synthesized many derivatives and analogues, including some rare amine derivatives which have been found in bacteria (such as hydroxyputrescine), nervous tissue (carboxyethylputrescine), and plants (carbamylputrescine). This tool offers the opportunity to define the amine patterns under varying conditions of growth and to look for unusual derivatives. We are currently studying the amine pattern in bacteria, cultured mammalian cells, transformed mammalian cells, and animal tissues. In view of the recent report on increased amine excretion in the urines of cancer patients, we are investigating the concentrations and patterns of amine excretion in the urines of normal individuals and cancerous patients.

(Drs. H. Tabor and C. W. Tabor)

Glutathionylspermidine is formed in *E. coli* during stationary phase, when the cells become anaerobic and the medium somewhat acid. All of the cellular spermidine and half of the glutathione are converted to this derivative. When such cells are placed under conditions favorable for growth, *i.e.*, fresh medium, pH 7.0, aerobiosis, the derivative is rapidly hydrolyzed to free spermidine and free glutathione. The structure of this compound has been determined by partial hydrolysis and characterization of the fragments; glutathione appears to be linked by an amide bond at glycine with a primary amine group of spermidine. We have quantitatively determined the spermidine using the technique developed above and the amino acids by standard analyzer techniques; one spermidine is present for each glutathione molecule. We are also surveying other sources for the presence of this derivative.

(Drs. C. W. Tabor and H. Tabor)

We have continued our investigations on the biosynthesis of spermidine with a very highly purified preparation of propylamine transferase from *E. coli*. We have found that although spermine is not formed in *E. coli*, this purified enzyme *in vitro* can carry out the synthesis of spermine as well as spermidine. Also, a propylamine derivative of cadaverine can be synthesized. Kinetic studies have been carried out with the pure enzyme.

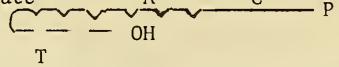
(Drs. W. H. Bowman, C. W. Tabor, and H. Tabor)

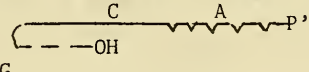
V. *PROTEIN AND NUCLEIC ACID*

Nucleic Acid Metabolism

The DNA polymerase induced by T4 bacteriophage has both polymerase and 3' to 5' exonuclease activities. We have determined the extent of hydrolysis of newly synthesized DNA by this exonuclease activity during DNA synthesis *in vitro*. In contrast to the DNA serving as template which is not degraded during synthesis, the hydrolysis of newly incorporated nucleotides occurs at a substantial rate and continues when there is no further accumulation of product DNA. While the base composition of newly synthesized DNA is determined by that of the template, the rate of hydrolysis of a newly incorporated nucleotide appears to depend on the recognition of a specific base by the polymerase. Thus with several different templates in which the rate and extent of net incorporation into product of the 4 deoxynucleotides were similar, the rate of hydrolysis of incorporated dAMP was 20-40% of its rate of net incorporation and was 1 1/2 to 3 times the rate of hydrolysis of dTMP or dGMP and 6 to 9 times that of dCMP.

Native duplex DNA does not serve as a template for net synthesis of DNA by the T4 polymerase *in vitro*, but the rate of incorporation and subsequent hydrolysis at the ends of native DNA is in fact greater than the rate of triphosphate utilization (net incorporation plus hydrolysis of newly incorporated residues) with single stranded DNA. When both native and single stranded DNA are present, the rates of both incorporation and hydrolysis are characteristic of that with single stranded DNA alone, suggesting a great preference of the enzyme for the latter substrate.

Most of the hydrolysis of newly incorporated residues occurs at the ends of fully copied chains, since hydrolysis of newly incorporated dTMP on a synthetic template  is much lower if dGTP is added to

allow further synthesis at the chain ends. However, there also appears to be some hydrolysis during the early stages of chain growth. With dGTP copying the C region of  , essentially all of the hydrolysis

results from turnover at fully copied chain termini.

An *in vitro* assay for DNA synthesis using as template the DNA present endogenously in extracts of T4 phage infected cells has been developed in order to be able to isolate and characterize the products of those genes known to be required for phage DNA synthesis whose functions are at present unknown. We are using this assay to purify the product of the phage gene 44.

(Drs. N. G. Nossal, M. S. Hershfield, and F. D. Gillin)

Protein Synthesis

The work on the soluble factors that promote the elongation of peptides on ribosomes has been significantly advanced since the last annual report. Highly purified, nearly homogeneous preparations have now been obtained and it can be tentatively concluded that two factors exist in the cytoplasm extracted from *E. coli*. These factors apparently can exist separate from each other or in one of two kinds of complexes: a rather loose complex that dissociates upon purification and a very stable complex that can be purified essentially to homogeneity. The roles of the complex and the separate factors in peptide chain elongation are now being studied *in vitro*. I have also prepared homogeneous preparations of phe $tRNA^{phe}$ and $tRNA^{phe}$ for use in the above experiments. This should enable the results to be interpreted in a much less ambiguous way than one could with crude tRNA. In conjunction with Dr. C. M. Cashel of the National Institute of Child Health and Human Development, an investigation of the role of these factors in the regulation of RNA synthesis has also been started.

(Dr. A. V. Furano)

3T3 and SV3T3 tRNA

The methylation of tRNA *in vitro* and *in vivo* was studied in a well controlled system consisting of normal mouse cells (3T3), and transformed mouse cells (SV3T3) which were obtained by transformation of the control 3T3 cells with SV40 virus. Cytoplasmic extracts prepared from transformed cells transferred methyl groups *in vitro* from S-adenosylmethionine to either *E. coli* B or mouse tRNA to a much greater extent than did extracts of nontransformed control cells. Furthermore, the tRNA methylating enzymes of the transformed cells had

an altered specificity which resulted in methylation of new tRNA sites in addition to those methylated by nontransformed cell enzymes.

The difference in the *in vitro* methylating activities of the two cell lines was not affected by dialysis or the addition of ammonium ion or polyamines. Furthermore, extracts of the nontransformed cells did not inhibit the methylating activity of the transformed cells.

In marked contrast to the results obtained *in vitro*, analysis of mouse tRNA methylated *in vivo* by growing cells in [^{14}C]methylmethionine or [^{3}H]-methylmethionine showed no detectable differences in the methylation pattern of nontransformed and transformed cellular tRNA. The extent of methylation and the methylated base composition of the two tRNA populations appeared to be similar. In addition, no differences were detected in the behavior on BD cellulose of intact methylated tRNA or on DEAE Sephadex of oligonucleotides of methylated tRNA. These two analytic techniques, however, were sensitive enough to detect differences in tRNA methylated *in vivo* by uninfected and T4 infected *E. coli* cells as well as tRNA methylated *in vitro* by transformed and nontransformed cells.

(Dr. M. Klagsbrun)

Ribonucleic Acid Metabolism

A new, unstable RNA fraction has been observed in extracts of *E. coli*. We are studying the nature of this material, using acrylamide gel electrophoresis, density gradient centrifugation, and isotopic methods. The preliminary evidence indicates that this material is smaller than 4S RNA and can be made to accumulate under special conditions of lysis.

(Dr. C. W. Tabor and Ms. P. D. Kellogg)

VI. *TRAUMATIC SHOCK AND TRANSPLANTATION*

Experiments on the effect of environmental temperature on burned hairless mice show that late mortality is significantly reduced in animals kept at 31° compared with 25°. These results may have important clinical implications in the treatment of burned patients. Studies on the mechanism of action of Na^+ on mortality of burned mice indicate that (1) the administration of Na^+ to normal and burned mice counteracts the toxic action of exogenous biogenic amines and (2) the amount of Na^+ in the bath of an *in vitro* preparation on guinea pig or rat uterus affects the contractile response of biogenic amines. These facts suggest that the action of Na^+ is primarily on receptor sites and secondarily on the circulation.

In experiments on cellular immunity, a preparation of phytohemagglutinin (PHA) purified for its mitogenic properties was tested in graft rejection and delayed hypersensitivity. Contrary to the results obtained with impure Difco PHA-P, the mitogenic fraction had no effect on cellular immunity. Intraperitoneal injection of N-acetyl-D-galactosamine or D-galactose was able to block partially the effect of PHA-P on the delayed hypersensitivity reaction. This

finding is the first evidence reported of such an action of simple sugars *in vivo* in cellular immunity.

(Dr. K. Markley, Mrs. E. T. Smallman, and Mr. S. W. Thornton)

The influence of increased and decreased local atmospheric pressure was studied on the effect of local swelling in the mouse tail. Following burn and tourniquet trauma the positive pressures that just decrease and totally inhibit swelling were established. In normal mice the subatmospheric pressures that cause edema were established, and the influence of age and of drugs was determined.

(Dr. S. M. Rosenthal and Mr. S. W. Thornton)

VII. *MYCOBACTERIUM*

Efforts were made to cultivate human blood monocytes as the host cell for the growth of *Mycobacterium leprae*. Although the monocytes could be maintained in fairly good condition for a period of 5 weeks, definite growth of *M. leprae* was not observed. Cytochemical studies on the growth of granulocytes in cultures of mouse bone marrow cells revealed that the colonies of granulocytes were grown on top of a "feeder cell" and that the "feeder cell" was a reticular cell. Motion picture studies on bone marrow macrophages revealed two interesting phenomena. One is the cyclic formation of large vacuoles in large macrophages followed by sudden popping of the vacuoles. The cyclic action of the vacuoles recurred for many hours, even days, and appeared to be a normal phenomenon of large macrophages. It is considered that such a phenomenon might represent a mechanical cleansing activity of the cell. The other phenomenon was the transformation of some large macrophages into elevated worm-like structures. The function of this action was obscure.

(Dr. Y. T. Chang and Mrs. R. N. Andersen)

VIII. *EVOLUTION*

A theory has been conceived for the origin of the genetic code. It is susceptible of experimental test and early experiments give it encouraging support.

(Dr. S. Black)

Serial No. NIAMD-LBP-1
1. Biochemical Pharmacology
2. Biochemistry of Amino Acids
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Biochemistry of Sulfur-Containing Compounds

Previous Serial Number: Same

Principal Investigator: Dr. Simon Black

Other Investigator: Mrs. Blondel Hazel

Cooperating Units: None

Man Years:

Total:	4.2
Professional:	2.0
Others:	2.2

Project Description:

Objectives:

A long term objective is discovery of enzymatic and regulatory mechanisms involved in the synthesis of constituents of living tissue, particularly of proteins.

Methods Employed:

Chemical and enzymatic techniques involving the use of spectrophotometry and radioactive substances are evolved to meet the needs of special problems as they arise.

Major Findings:

I. Protein Synthesis. The glycyl tRNA synthetase from yeast, an essential enzyme in protein biosynthesis, catalyzes the following reaction:

$$\text{glycine} + \text{ATP} + \text{tRNA} \rightleftarrows \text{glycyl tRNA} + \text{AMP} + \text{PP}.$$

It has been found previously that when this enzyme is tested in the presence of inorganic sulfide, its activity becomes completely dependent upon two activator-like substances: glutathione and an alkali-denatured protein.

It is our hypothesis that the denatured protein acts as a substitute for a specific activator present in the cell, and efforts have been directed to developing a test which will distinguish between this activator and non-specific ones. During the past year a practical test for such a specific substance has been further perfected, and procedures for its purification and characterization have been developed.

II. Evolution. A theory has been conceived for the origin of the genetic code. It is susceptible of experimental test, and early experiments give it encouraging support.

Significance to Biomedical Research and the Program of the Institute:

I. Protein Synthesis. Elucidation of the nature of the postulated activator and its interaction with the enzyme would open to similar study the regulation of many other of the more complex anabolic reactions of the cell, which are thought to be related to the functions of the hormones.

II. Evolution. It is hoped that a plausible theory for the origin of living organisms, supported by observation and experiment, will throw light on the nature of fundamental chemical interactions in present-day cells, and that this will be useful in enlarging our understanding and control of disease processes.

Proposed Course:

I. Protein Synthesis. Studies will continue toward eventual isolation and characterization of the enzymatic activator substance.

II. Evolution. Theoretical and experimental work are being pursued with a view to determining how the first proteins and nucleic acids arose in nature, and how the genetic code originated.

Honors and Awards: None

Publications:

Black, S.: A hypothesis and an experiment on the origin of the genetic coding process. *Biochem. Biophys. Res. Commun.* 43: 267-272, 1971.

Serial No. NIAMD-LBP-2
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Chemotherapy of Mouse Leprosy

Previous Serial Number: Same

Principal Investigator: Dr. Yao Teh Chang

Other Investigator: Mrs. Roxanna N. Andersen

Cooperating Unit: Leonard Wood Memorial (The American Leprosy Foundation)

Man Years:

Total:	3.1
Professional:	2.0
Others:	1.1

Project Description:

Objectives:

The evaluation of therapeutic effectiveness of drugs in mouse leprosy. The tissue cultures of intracellular parasites.

Methods Employed:

Chemotherapeutic studies were performed in mice infected intraperitoneally or into foot pads with *Mycobacterium lepraemurium*. Further attempts to cultivate *M. leprae* were made using cultures of human blood macrophages and macrophages obtained from mice which had been thymectomized and irradiated with 950 rads. Cytochemical and cinemicrographic studies of granulocytes and macrophages of mouse bone marrow cells were performed in cultures in Leighton tubes and Sight chambers.

Major Findings:

1. *Cultivation trial of Mycobacterium leprae in macrophages obtained from thymectomized-irradiated mice.* Thymectomized-irradiated mice were obtained from the Oak Ridge National Laboratory monthly for 6 months. Macrophages obtained from these mice, either from the peritoneal cavity, from bone marrow, or from both, were infected with *M. leprae*. The cultures were maintained at 37° and 30°. A total of 9 biopsies were received from the Philippines and

Surinam. Cultures infected with these biopsy materials were maintained for periods of a few weeks to 10 months. No growth of the organisms was seen.

2. *Attempts to establish human blood macrophages as Mycobacterium leprae host cells.* Despite our efforts to find the suitable medium for growth of blood macrophages, the cell system is still far from satisfactory. Blood macrophages can be maintained in fairly good condition for only a short period of time, *e.g.*, 5 weeks. Since long-term maintenance of host cells appears to be the chief requirement for growth of *M. leprae*, the leprosy bacilli inoculated into these cells showed only signs of degeneration.

3. *Effect of DDS (4,4'-diaminodiphenylsulfone) against Mycobacterium lepraemurium in mouse foot pads.* Growth of *M. lepraemurium* in mouse foot pads was studied in a total of 16 inbred strains of mice. Marked variations of bacterial growth was observed not only among different strains but also among the individual animals of the same strain. Among the 16 strains the DBA and $AALF_1$ strains revealed the least animal-to-animal variation. These two strains of mice were selected to study the activity of various doses of DDS. The experiments were terminated but the bacillary counts are not completed.

4. *Cytochemical studies of mouse bone marrow cells in cultures.* Mouse bone marrow gave rise to cell colonies which appeared to grow on top of a feeder cell *in vitro*. The cytochemical characteristics of the cells in the colony were studied. The colony cells were positive for myeloperoxidase and Sudan black B, while the feeder cell was positive for acid phosphatase, alkaline phosphatase, PAS, and nonspecific esterase. The colony cells were mostly of the myelocytic series, and the feeder cell was apparently a reticular cell. Only a few macrophages were occasionally observed in the colony. It is probable that they migrated from outside instead of being generated within the colony.

5. *Motion picture studies of cell colonies in bone marrow cultures.* Cells in the colony showed continuous cell division and migration of the cells outside the colony. All the cells were gone in a period of 3 to 4 days. The feeder cell showed an ameboid movement. A single colony occasionally contained more than one feeder cell which sometimes separated the colony into several small colonies. Death of cells was also observed within the colonies. Macrophages were often observed to enter and leave the colony.

6. *Motion picture studies of mouse bone marrow macrophages.* The life history of mouse bone marrow macrophages was filmed with 16 mm motion pictures. Their reproduction, death, phagocytosis, garbage removal, pinocytosis, bleb formation, and a worm-like movement have been recorded in an 18 minute move. One interesting phenomenon was the cyclic formation of large vacuoles which subsequently collapsed. This cyclic action of vacuoles recurred for many hours, even days, and appeared to be a normal phenomenon of large macrophages. It is considered that such a phenomenon might represent a mechanical cleansing activity of the cell.

Significance to Biomedical Research and the Program of the Institute:

Considering the unsuccessful past trials of growing *M. leprae* in various types of cells, including the macrophages of animals, it seems reasonable that the most probable host cell for *M. leprae* would be macrophages of humans, the natural host cell of leprosy infection. Although the maintenance of human macrophages has been successful for only a short period of time, it is hoped a suitable technique can be worked out, especially with the human bone marrow biopsies. The cytochemical and motion picture studies of bone marrow cells offer an opportunity to study the characteristics of macrophages in dealing with the intracellular parasites. This will lead to a better understanding of the host-parasite relationship which is of importance in respect of all intracellular parasitic diseases.

Proposed Course:

Studies will be continued toward the development of techniques for cultivation of human macrophages, on the growth of *M. leprae* therein, on cytochemical and cinemicrographic studies of host-parasite relationship, in *in vitro* granulocytopoiesis, and on development of a more accurate model for drug screening in murine leprosy.

Honors and Awards: None

Publications:

Chang, Y. T., and Andersen, R. N.: Cultivation of mouse bone marrow cells. *J. Reticuloendothelial Soc.* 9: 568-579, 1971.

Serial No. NIAMD-LBP-3
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies in Traumatic Shock and Cellular Immunity

Previous Serial Number: Same

Principal Investigator: Dr. Kehl Markley

Other Investigators: Dr. Sanford M. Rosenthal, Mrs. Elizabeth T. Smallman, and Mr. Steven W. Thornton

Cooperating Units: None

Man Years

Total:	4.0
Professional:	3.5
Other:	0.5

Project Description:

Objectives:

(1) To study the effects of environmental temperature on experimental burn mortality.

(2) To study the effect of Na^+ on biogenic amines.

(3) To study the mechanism of action of phytohemagglutinin (PHA) on cellular immunity.

(4) To study the effect of altered tissue pressures on the production of edema.

Methods Employed:

(1) Hairless mice were given a 2/3 body surface area burn at 70° for 5-8 seconds and placed at environmental temperatures of 4°, 25°, 31°, and 37°. Mortality was recorded for 48 hours for shock mortality and from 2-21 days for late mortality. Measurements were made of body weight, food and water intake, and rectal temperatures during this time period in order to estimate metabolic changes.

(2) *In vivo Effect of* Na^+. Normal, NIH mice were given toxic doses of histamine, serotonin, and ATP intraperitoneally. Forty-eight hour mortality was studied and compared with mice given fluid therapy in addition to the toxic amines. Fluid therapy consisted of 15% body weight of 5% glucose in water or 0.85% NaCl. Other mice were made hyponatremic by giving them a 2/3 body surface area burn at 70° for 6 seconds. The effect of fluid therapy was tested again after the administration of the same toxic amines.

In vitro Effect of Na^+. The uterus of rats or guinea pigs in estrus was removed and placed in a bath of de Jalon's solution at 31°. Contractions of the muscles were recorded when the following amines were added to the bath: bradykinin, histamine, serotonin, ATP, and epinephrine. The effect of varying the Na^+ concentration or osmolarity of the bath solution on the contraction pattern was measured in the presence or absence of the amines.

(3) PHA-P (Difco) was boiled to destroy its mitogenic and erythroagglutinating properties without significantly changing its immunogenic properties. A purified mitogenic fraction of PHA was isolated by the technique of Goldberg, Rosenau, and Burke (*Proc. Nat. Acad. Sci. U.S.A.* 64: 283, 1969). These preparations, together with the Difco PHA-P, were tested on skin allograft survival time in inbred mice and the delayed hypersensitivity reaction in guinea pigs sensitized to *M. tuberculosis*. In some experiments simple sugars were administered intraperitoneally to animals to test their ability to block the reaction of PHA on both types of cellular immunity.

(4) Methods of altering tissue pressure in the mouse tail were devised by subjecting the tail to increased or decreased atmospheric pressure. The influence of age, hormones, and drugs on the swelling produced by decreased atmospheric pressure was studied. The critical pressure for swelling in trauma was determined by the response to graded increases of pressure.

Major Findings:

Experiments on the effect of environmental temperature on burned hairless mice show that late mortality is significantly reduced in animals kept at 31° compared with 25°. Studies on the effect of Na^+ on biogenic amines indicate that (1) the administration of Na^+ to normal and burned mice counteracts the toxic action of exogenous biogenic amines and (2) the amount of Na^+ in the bath of an *in vitro* preparation on guinea pig or rat uterus affects the contractile response of biogenic amines.

In experiments on cellular immunity, a preparation of PHA purified for its mitogenic properties was tested in graft rejection and delayed hypersensitivity. Contrary to the results obtained with impure Difco PHA-P, the mitogenic fraction had no effect on cellular immunity. Intraperitoneal injection of N-acetyl-D-galactosamine or D-galactose was able to block partially the effect of PHA-P on the delayed hypersensitivity reaction. This finding is the first evidence reported of such an action of simple sugars *in vivo* in cellular immunity.

The influence of increased and decreased local atmospheric pressure was studied on the effect of local swelling in the mouse tail. Following burn and

tourniquet trauma the positive pressures that just decrease and totally inhibit swelling were established. In normal mice the subatmospheric pressures that cause edema were established, and the influence of age and of drugs was determined.

Significance to Biomedical Research and the Program of the Institute:

The studies on the effect of environmental temperature on burned hairless mice may have important clinical implications on the treatment of burned patients. The *in vivo* and *in vitro* experiments on the action of Na^+ on biogenic amines suggest that the action of Na^+ is primarily on receptor sites and secondarily on the circulation. The results obtained from the studies on the mechanism of action of PHA on cellular immunity suggest that its mitogenic or immunogenic properties alone are not responsible for its action. The experiments on tissue pressures in normal and traumatized mice have significance in the understanding of edema formation.

Proposed Course:

(1) To continue the study of the action of Na^+ on the mechanism of shock.

(2) To study the physical and chemical properties of the purified PHA.

(3) To continue the investigation on the mechanism of swelling after trauma.

Honors and Awards: None

Publications:

Markley, K.: Vaccine prophylaxis for pseudomonas infections. *Ann. Intern. Med.* 74: 140, 1971.

Markley, K., Smallman, E., and Thornton, S. W.: Demonstration of a circulating endotoxin hapten in burned mice. *Proc. Soc. Exp. Biol. Med.* 137: 584-589, 1971.

Markley, K., Smallman, E., and Thornton, S. W.: Protection against burn, tourniquet and endotoxin shock by histamine, 5-hydroxytryptamine and 5-hydroxytryptamine derivatives. *Brit. J. Pharmacol.* 42: 13-24, 1971.

Markley, K., Thornton, S. W., and Smallman, E.: The effect of traumatic and nontraumatic shock on allograft survival. *Surgery* 70: 667-673, 1971.

Markley, K., Thornton, S. W., and Smallman, E.: On the mechanism of action of phytohemagglutinin in cellular immunity. *Proc. Soc. Exp. Biol. Med.* 139: 37-42, 1972.

Serial No. NIAMD-LBP-4
1. Biochemical Pharmacology
2. Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Biology of Complex Carbohydrates

Previous Serial Number: Same

Principal Investigator: Dr. Victor Ginsburg

Other Investigators: Drs. Akira Kobata, Chaviva Isersky-Carter, and David A. Zopf

Cooperating Units: None

Man Years:

Total:	4.5
Professional:	2.8
Others:	1.7

Project Description:

Objectives:

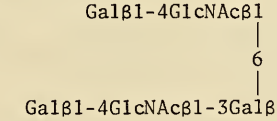
To investigate the role of complex carbohydrates in cell biology.

Methods Employed:

Usual biochemical techniques.

Major Findings:

A new hexasaccharide, "lacto-*N*-*neo*hexaose", has been isolated from human milk and its structure determined:



In addition, several fucosyl and sialyl derivatives of lacto-*N*-*neo*hexaose and the previously described hexaose, lacto-*N*-hexaose, have been partially characterized.

Malignant transformation of cells is accompanied by alterations in their surface sugars which can lead to alterations in serologic properties. For example, viral-transformed 3T3 cells (mouse fibroblasts) have 50% less sialic acid than normal 3T3 cells. It was not known whether the observed reduction in sialic acid was due to loss of sialic acid in a particular linkage or to a general loss of sialic acid in all linkages. Sialyl linkages are more resistant to acetolysis than are hexosyl or *N*-acetylhexosaminyl linkages and disaccharides containing sialic acid linked to hexoses or *N*-acetylhexosamine can be isolated from the acetolysate. Cells were grown in [^{14}C]glucosamine in order to label their sialic acid. After extraction with TCA, the cell residues were acetolyzed. Disaccharides containing sialic acid were isolated using ion-exchange columns and paper chromatography and their ^{14}C activity determined. With 3T3 mouse cells approximately equal, ^{14}C activity was found associated with the standard disaccharides, NANA-α-2-3-Gal and NANA-α-2-6-Gal; no activity was associated with sailyl disaccharides containing *N*-acetylhexosamine. No change in ratio was found using 3T3 cells transformed with SV40 or MSV viruses. Therefore, the decrease in sialic acid after transformation reported by others [*cf.*, Ohta *et al.*, *Biochim. Biophys. Acta*, 158, 98 (1968) and Wu *et al.*, *Biochemistry*, 9, 5083 (1970)] results from a proportional decrease in both types of linkages.

A new method has been worked out to couple oligosaccharides to proteins in order to prepare synthetic antigens of known structures.

Significance to Biomedical Research and the Program of the Institute:

These studies may contribute to an understanding of cell-cell interactions which are important to biological phenomena such as graft rejection, autoimmune diseases, and carcinogenesis.

Proposed Course:

We will continue to try to elucidate the carbohydrate structures on cell surfaces that are responsible for their specificity.

Honors and Awards: None

Publications:

Ginsburg, V.: Enzymatic basis for blood types in man. In Meister, A. (Ed.): *Advances in Enzymology*, Vol. 36. New York, New York, Wiley, 1972, in press.

Ginsburg, V.: Isolation of sugar nucleotides. In Whistler, R. L., and Bemiller, J. N. (Eds.): *Methods in Carbohydrate Chemistry*, Vol. 6. New York, New York, Academic Press, 1972, pp. 433-435.

Ginsburg, V., and Kobata, A.: Structure and function of surface components of mammalian cells. In Rothfield, L. (Ed.): *Structure and Function of Biological Membranes*. New York, New York, Academic Press, 1971, pp. 439-459.

Ginsburg, V., Kobata, A., Hickey, C., and Sawicka, T.: Biochemical basis for blood types in man. In Jamieson, G. A., and Greenwalt, T. J. (Eds.): *Glycoproteins of Blood Cells and Plasma*. Philadelphia, Pennsylvania, Lippincott, 1971, pp. 114-126.

Kobata, A.: An *N*-acetylgalactosaminyltransferase from human milk; a product of the gene that determines blood type A. In Ginsburg, V. (Ed.): *Methods in Enzymology*, Vol. 18. New York, New York, Academic Press, *in press*.

Kobata, A.: Isolation of oligosaccharides from human milk. In Ginsburg, V. (Ed.): *Methods in Enzymology*, Vol. 18. New York, New York, Academic Press, *in press*.

Kobata, A., and Ginsburg, V.: Oligosaccharides of human milk. III. Isolation and characterization of a new hexasaccharide, lacto-*N*-hexaose. *J. Biol. Chem.* 247: 1525-1529, 1972.

Kobata, A., and Ginsburg, V.: Oligosaccharides of human milk. IV. Isolation and characterization of a new hexasaccharide, lacto-*N*-*neo*hexaose. *Arch. Biochem. Biophys.*, *in press*.

Shen, L. C., and Ginsburg, V.: Sugar analysis of cells in culture by isotope dilution. In Ginsburg, V. (Ed.): *Methods in Enzymology*, Vol. 18. New York, New York, Academic Press, *in press*.

Serial No. NIAMD-LBP-5A
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Estimation, Metabolism, and Function of Amines

Previous Serial Number: Same

Principal Investigators: Drs. Herbert Tabor and Celia White Tabor

Other Investigators: Dr. William H. Bowman and Mrs. Patricia Deal Kellogg

Cooperating Units: Dr. F. Irreverre, Laboratory of Biophysical Chemistry, National Institute of Arthritis and Metabolic Diseases

Man Years:

Total:	4.7
Professional:	3.2
Others:	1.5

Project Description:

Objectives:

The importance of the various polyamines is shown by their wide distribution in viruses, bacteria, plant cells, and animal cells. Studies are being conducted to elucidate their metabolism and function.

Methods Employed:

The amines and their derivatives are studied by isotopic labeling and special chromatographic techniques. Standard enzymological techniques have been used to purify further the enzymes that synthesize and degrade spermine, spermidine, and monoamines. Chemical synthetic procedures have been utilized to prepare derivatives for identification of enzymatic products. Uptake of amines was studied by counting the residual radioactivity in washed pellets on Millipore filters. Gel electrophoresis techniques have been utilized to study the ribosome pattern of cells. Amino acid analyses were carried out in an automatic amino acid analyzer. Fluorometric analyses have been utilized.

An amino acid analyzer has been utilized for the development of a new, automated, sensitive, and quantitative method for the analysis of amines and amine derivatives.

Major Findings:

With Dr. F. Irreverre of this Institute, we have developed a sensitive, automated method for the analysis of amines and amine derivatives with an amino acid analyzer. This is the first method available which is both sensitive and sufficiently specific to permit complete separation of closely related amines. We have synthesized many derivatives and analogues, including some rare amine derivatives which have been found in bacteria (such as hydroxyputrescine), nervous tissue (carboxyethylputrescine), and plants (carbamylputrescine). This tool offers the opportunity to define the amine patterns under varying conditions of growth and to look for unusual derivatives. We are currently studying the amine pattern in bacteria, cultured mammalian cells, transformed mammalian cells, and animal tissues. In view of the recent report on increased amine excretion in the urines of cancer patients, we are investigating the concentrations and patterns of amine excretion in the urines of normal individuals and cancerous patients.

Glutathionylspermidine is formed in *E. coli* during stationary phase, when the cells become anaerobic and the medium somewhat acid. All of the cellular spermidine and half of the glutathione are converted to this derivative. When such cells are placed under conditions favorable for growth, *i.e.*, fresh medium, pH 7.0, aerobiosis, the derivative is rapidly hydrolyzed to free spermidine and free glutathione. The structure of this compound has been determined by partial hydrolysis and characterization of the fragments; glutathione appears to be linked by an amide bond at glycine with a primary amine group of spermidine. We have quantitatively determined the spermidine using the technique developed above and the amino acids by standard analyzer techniques; one spermidine is present for each glutathione molecule. We are also surveying other sources for the presence of this derivative.

We have continued our investigations on the biosynthesis of spermidine with a very highly purified preparation of propylamine transferase from *E. coli*. We have found that although spermine is not formed in *E. coli*, this purified enzyme *in vitro* can carry out the synthesis of spermine as well as spermidine. Also, a propylamine derivative of cadaverine can be synthesized. Kinetic studies have been carried out with the pure enzyme.

A new, unstable RNA fraction has been observed in extracts of *E. coli*. We are studying the nature of this material, using acrylamide gel electrophoresis, density gradient centrifugation, and isotopic methods. The preliminary evidence indicates that this material is smaller than 4S RNA and can be made to accumulate under special conditions of lysis.

Significance to Biomedical Research and the Program of the Institute:

Recent work has indicated that the polyamines may play an important role in permeability of organisms and in the stability of nucleic acids inside cells. Recent studies by Pardee and coworkers on synchronous cultures of *E. coli* suggest that the amines may also play an important role in the division of *E. coli*.

Some of the polyamines have also been found to act as growth factors for bacteria, plants, and certain mammalian cell lines in culture. Our studies of the amine content and metabolism in bacteria and mammalian cells under various growth conditions will contribute to our understanding of the functions of these ubiquitous compounds.

Our studies on the propylamine transferase enzyme may lead to elucidation of the mechanism of the formation of the C-N bond in bacteria and the role of the high-energy sulfonium ion in this reaction.

Proposed Course:

Further studies on the metabolism and function of the biologically important amines. We hope to learn the normal physiological function of these amines in the cell and to explain such pathological effects as the production of renal damage.

Honors and Awards: None

Publications:

Tabor, H., and Tabor, C. W.: Biosynthesis and metabolism of 1,4-diaminobutane, spermine, spermidine, and related amines. In Meister, A. (Ed.): *Advances in Enzymology and Related Areas of Molecular Biology*, Volume 35. New York, John Wiley & Sons, in press.

Serial No. NIAMD-LBP-5B
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Formiminoglutamate Iminohydrolase

Previous Serial Number: Same

Principal Investigators: Drs. Reed B. Wickner and Herbert Tabor

Other Investigators: None

Cooperating Units: None

Man Years

Total:	0.0
Professional:	0.0
Other:	0.0

This project has been discontinued.

Publications:

Wickner, R. B., and Tabor, H.: N-Formimino-L-glutamate iminohydrolase from histidine-adapted *Pseudomonas*. *J. Biol. Chem.* 247: 1605-1609, 1972.

Serial No. NIAMD-LBP-6
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Enzymatic Studies of Nucleic Acid Metabolism

Previous Serial Number: Same

Principal Investigator: Dr. Nancy G. Nossal

Other Investigators: Dr. Michael S. Hershfield
Dr. Frances D. Gillin

Cooperating Units: None

Man Years:

Total:	2.8
Professional:	2.3
Others:	0.5

Project Description:

Objectives:

To determine the characteristics and functions of enzymes which are involved in the synthesis, modification, and degradation of DNA.

Methods Employed:

Standard biochemical techniques were employed for the preparation of polynucleotide substrates and enzymes. Extensive use has been made of amber mutants of T4 bacteriophage which are unable to make specific early enzymes in nonpermissive hosts. Procedures have been developed for the large scale preparation of these amber mutants and for the production of kilogram quantities of infected bacterial cells.

Major Findings:

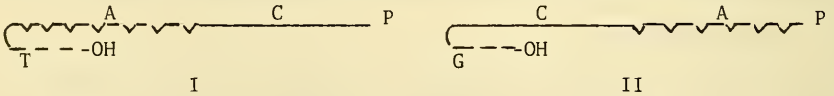
The DNA polymerase induced by T4 bacteriophage has both polymerase and 3' to 5' exonuclease activities. In studies designed to elucidate the role of this exonuclease activity during DNA synthesis, we showed previously that while the DNA serving as template is protected from degradation by the presence of the deoxynucleoside triphosphates required for synthesis, there is

extensive hydrolysis of newly incorporated nucleotides. We have now examined the parameters controlling the degradation of newly synthesized DNA, using thin layer chromatography to follow simultaneously nucleotide incorporation, hydrolysis of newly synthesized DNA (mononucleotides derived from the triphosphates used for polymerization), and degradation of template DNA.

During copying of single stranded DNA, hydrolysis of newly incorporated residues occurs at a rate slower than, but related to, the initial rate of synthesis and continues after product DNA has ceased to accumulate. For example, at 37° the rate of hydrolysis of newly incorporated dAMP is 20% to 40% of the rate of dAMP incorporation. The polymerase appears to recognize specific bases since in a reaction in which the four deoxynucleotides are incorporated into product DNA at similar rates and to the same extent, newly added dAMP is hydrolyzed 1 1/2 to 3 times as often as dTMP and dGMP and 6 to 9 times more frequently than dCMP. At 20° the rate of hydrolysis of newly incorporated residues is depressed to a greater degree than the rate of synthesis, suggesting that local unwinding of the 3' terminus is required for the hydrolysis of newly incorporated residues.

Native duplex DNA does not serve as a substrate for net DNA synthesis by the T4 polymerase *in vitro*. Limited incorporation and subsequent hydrolysis at the ends of native DNA does occur and triphosphate utilization (net incorporation plus hydrolysis of newly incorporated residues) is in fact faster with native than with single stranded DNA. However, when both native and single stranded DNA are present, the rate of both incorporation and hydrolysis are characteristic of that seen with single stranded DNA alone, suggesting a preference of the enzyme for single stranded DNA.

In order to determine how much of the hydrolysis of newly added residues goes on during chain growth, and how much at the ends of fully copied molecules, we have in collaboration with Dr. F. J. Bollum, University of Kentucky College of Medicine, made deoxy-homopolymers of the following types:



Hydrolysis of newly incorporated dTMP on polymer I is much lower in the presence of dGTP showing that most of the hydrolysis occurs at chain ends. However, there also appears to be some hydrolysis during the early stages of chain growth. With dGTP copying the C regions of polymer II, essentially all of the hydrolysis results from turnover at the ends of fully copied molecules.

The T4 DNA polymerase is required for phage DNA replication *in vivo* since mutants in the polymerase structural gene do not make DNA. Several other genes must also be functioning for phage DNA to be made. In order to be able to isolate and characterize the products of those genes required for DNA synthesis whose functions are at present unknown, we have developed an *in vitro* assay for DNA synthesis using as template the DNA present endogenously in extracts of infected cells. Although the rate of synthesis in

this *in vitro* system is low, it can be used as an assay for factors required for phage DNA synthesis. Extracts of cells infected with phage defective in DNA synthesis do not make DNA in this system, but incorporation is increased by mixing extract each missing the product of one required gene. We are presently using this method to purify the product of the phage gene 44.

Significance to Biomedical Research and the Program of the Institute:

Knowledge of enzymes controlling DNA synthesis and degradation is important to an understanding of the control of DNA replication and transcription in both normal and viral infected cells.

Proposed Course:

The product of the phage gene 44 will be purified and its function in DNA synthesis studied. In order to try to understand how mutations in the structural gene of the T4 DNA polymerase lead to increased or decreased rates of mutation, we will compare the rate of incorporation and subsequent hydrolysis of noncomplementary nucleotides (copying errors) by purified DNA polymerases from the wild type phage and from polymerase mutants known to have altered frequencies of mutation. Possible changes in the level of cyclic AMP or in adenyl cyclase occurring as a result of phage infection and during sporulation of *B. subtilis* will be investigated.

Honors and Awards: None

Publications:

Nossal, N. G., and Hershfield, M. S.: Nuclease activity in a fragment of bacteriophage T4 deoxyribonucleic acid polymerase induced by the amber mutant *am* B22. *J. Biol. Chem.* 246: 5414-5426, 1971.

Hershfield, M. S., and Nossal, N. G.: Hydrolysis of template and newly synthesized deoxyribonucleic acid by the 3' to 5' exonuclease activity of the T4 deoxyribonucleic acid polymerase. *J. Biol. Chem.* 247, issue of June 10, 1972, in press.

Serial No. NIAMD-LBP-7
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Protein Synthesis in *Escherichia coli*

Previous Serial Number: Same

Principal Investigator: Dr. Anthony V. Furano

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1.1
Professional:	1.0
Others:	0.1

Project Description:

Objectives:

To study the mechanism and control of protein synthesis *in vivo* and *in vitro*.

Methods Employed:

In vivo. We have been able to isolate and begin to study a mutant of *E. coli* which cannot synthesize protein at 42°.

In vitro. We have been investigating the functional capacities of ribosomes that have been modified either by treatment with a selective chemical reagent or by genetic alteration. The modified ribosomes are tested for their ability to participate in the various stages of protein synthesis *in vitro*: *i.e.*, chain initiation, chain elongation, chain termination. We are also studying the soluble enzymes that catalyze in some way polypeptide chain elongation.

Major Findings:

The work on the soluble factors that promote the elongation of peptides on ribosomes has been significantly advanced since the last annual report.

Highly purified, nearly homogeneous preparations have now been obtained and it can be tentatively concluded that two factors exist in the cytoplasm extracted from *E. coli*. These factors apparently can exist separate from each other or in one of two kinds of complexes: a rather loose complex that dissociates upon purification and a very stable complex that can be purified essentially to homogeneity. The roles of the complex and the separate factors in peptide chain elongation are now being studied *in vitro*. I have also prepared homogeneous preparations of phe $tRNA^{phe}$ and $tRNA^{phe}$ for use in the above experiments. This should enable the results to be interpreted in a much less ambiguous way than one could with crude tRNA. In conjunction with Dr. C. M. Cashel of the National Institute of Child Health and Human Development, an investigation of the role of these factors in the regulation of RNA synthesis has also been started.

Significance to Biomedical Research and the Program of the Institute:

Knowledge on the mechanism of protein synthesis is essential to understanding cellular metabolism and growth.

Proposed Course:

This work is currently in progress and will be continued and extended.

Honors and Awards: None

Publications:

Furano, A. V.: A very rapid method for washing large numbers of precipitates of proteins and nucleic acids. *Anal. Biochem.* 43: 639-640, 1971.

Furano, A. V.: Small-scale purification of ^{35}S-glutathione. In Tabor, H., and Tabor, C. W. (Eds.): *Methods in Enzymology*, Volume 17, Part B. New York, Academic Press, 1971, pp. 509-510.

Furano, A. V., and Harris, M. I.: The activity of ribosomes whose RNA has been degraded by incubation in the presence or absence of oxidized glutathione. *Biochim. Biophys. Acta* 247: 291-303, 1971.

Serial No. NIAMD-LBP-8
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Methylation of Transfer RNA in Normal and Transformed Cells

Previous Serial Number: Same

Principal Investigator: Dr. Michael Klagsbrun

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1.1
Professional:	1.0
Other:	0.1

Project Description:

Objectives:

To determine whether the enhanced tRNA methylating capacity of transformed cells compared to normal cells found *in vitro* has any biological significance *in vivo*.

Methods Employed:

Standard tissue culture and biochemical techniques such as column and thin layer chromatography, sucrose gradients, gel electrophoresis, and autoradiography.

Major Findings:

The methylation of tRNA *in vitro* and *in vivo* was studied in a well controlled system consisting of normal mouse cells (3T3), and transformed mouse cells (SV3T3) which were obtained by transformation of the control 3T3 cells with SV40 virus. Cytoplasmic extracts prepared from transformed cells transferred methyl groups *in vitro* from S-adenosylmethionine to either *E. coli* B or mouse tRNA to a much greater extent than did extracts of nontransformed control cells. Furthermore, the tRNA methylating enzymes of the transformed cells had an altered specificity which resulted in methylation of new tRNA sites in addition to those methylated by nontransformed cell enzymes.

In marked contrast to the results obtained *in vitro*, analysis of mouse tRNA methylated *in vivo* by growing cells in [^{14}C]methylmethionine or [^{3}H]-methylmethionine showed no detectable differences in the methylation pattern of nontransformed and transformed cellular tRNA. The extent of methylation and the methylated base composition of the two tRNA populations appeared to be similar. In addition, no differences were detected in the behavior on BD cellulose of intact methylated tRNA or on DEAE Sephadex of oligonucleotides of methylated tRNA. These two analytic techniques, however, were sensitive enough to detect differences in tRNA methylated *in vivo* by uninfected and T4 infected *E. coli* cells as well as tRNA methylated *in vitro* by transformed and nontransformed cells.

Significance to Biomedical Research and the Program of the Institute:

It has been claimed that cytoplasmic extracts of tumor cells with a viral (SV40, adeno 12) origin, have much higher methylase activities than do extracts of normal cell controls. As a result possible differences might exist in the structure of the tRNA of tumor and normal cells, and these differences could play a role in the regulation of protein synthesis in these types of cells.

Proposed Course:

The discrepancies between the *in vitro* and *in vivo* results which are obtained when comparing methylation of tRNA in normal and transformed cells will be explored. In particular, *in vitro* methylation will be studied to determine the effects of contact inhibition and growth on methylase activity.

Honors and Awards: None

Publications:

Klagsbrun, M.: Changes in the methylation of transfer RNA in vaccinia infected HeLa cells. *Virology* 44: 153-167, 1971.

Serial No. NIAMD-LBP-9
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on the Chemical and Physiological Properties of the Surface of *Escherichia coli*

Previous Serial Number: Same

Principal Investigator: Dr. Loretta Leive

Other Investigators: Drs. Charles F. Kulpa, Jr., and David C. Morrison

Cooperating Units: None

Man Years:

Total:	2.3
Professional:	2.0
Other:	0.3

Project Description:

Objectives:

To study the chemical and physiological properties of the cell surface of *E. coli*.

Methods Employed:

Usual biochemical methods, including isolation and purification of cell surface components by extraction, ultracentrifugation, chromatography, and selective enzymatic degradation, determination of the size and shape of these fractions by biophysical measurements of density, viscosity, and ultracentrifugal properties, identification and quantitation of sugars by colorimetric and enzymatic procedures, and use of labeled compounds to specifically label cell fractions *in vivo*.

Major Findings:

The surface of *E. coli* consists of an inner, or cytoplasmic, membrane, a rigid cell wall, and an outer membrane. Recent work in another laboratory has indicated that lipopolysaccharide (LPS) is synthesized in the cytoplasmic membrane and then transferred to the outer membrane. We wanted to determine

whether such newly completed LPS molecules enter into discrete specialized regions of the outer membrane or whether they are inserted randomly throughout this membrane. We used a mutant of *E. coli* which forms LPS rich in carbohydrate when grown with galactose but forms LPS with half as much carbohydrate when grown without galactose. Since the density of the outer membrane increases with increasing carbohydrate content, fragments of outer membrane from cells grown in the presence of galactose are denser than fragments of outer membrane from cells grown in its absence. These two types of membrane fragments can be separated by equilibrium centrifugation. Cells were therefore grown in the absence of galactose and galactose then added. At various times thereafter membranes were prepared, fragmented by sonication, and centrifuged to equilibrium on sucrose. Within 4 minutes of exposure to galactose, equivalent to 10% of a doubling time, a small percent of the outer membrane fragments were found to have the very high density characteristic of fragments from galactose-grown cells. This result is consistent with the hypothesis that newly synthesized LPS molecules enter the outer membrane in discrete, specialized regions. The results are incompatible with entry randomly throughout the existing membrane.

Other experiments have been directed toward better definding the composition and structure of LPS. Although the overall composition and biosynthesis of LPS from different coliform bacteria is well known, there has been little information available on whether the LPS of a given organism consists of a homogeneous population of molecules or whether there are fractions of differing composition. The LPS of one strain of *E. coli*, 0111:B4, has been isolated by two methods: the classical phenol extraction method and a new aqueous butanol procedure devised in this laboratory. Material isolated by both methods shows two separable fractions that have distinct composition, and distinct physical characteristics as indicated by equilibrium centrifugation, sedimentation, diffusion, and viscosity. One fraction is a long rigid molecule with a very high proportion of antigenic carbohydrate side chains. The other fraction is more nearly globular, shows a tendency to aggregate, and has a lower proportion of antigenic side chains.

Significance to Biomedical Research and the Program of the Institute:

Study of the organization of the outer layer of the cell surface will contribute to understanding its structure and its interaction with the environment. In addition, earlier work from this laboratory showed that the outer layer is the barrier to many charged molecules in coliform bacteria, including many drugs, and that this barrier can be reduced by exposing the cells to EDTA, thus making them drug-sensitive. The current studies are aimed at understanding the structural basis for this permeability barrier, and such understanding may have implications for drug therapy.

Proposed Course:

The specialized regions of LPS entry mentioned above will be isolated, in order to determine their size, the number of them per cell, and their composition. It will be of interest to determine whether they are different in composition from the bulk of the outer membrane, since such a finding would be one of the first demonstrations of chemical heterogeneity in a morphologically

uniform membrane bilayer structure. The rate of diffusion of LPS from these points to the rest of the outer membrane will also be measured. Mutants requiring diaminopimelic acid, a constitutent of cell wall, or requiring unsaturated fatty acids, needed for surface lipids, will be isolated from the *E. coli* mutant used in the above studies. Diaminopimelic acid auxotrophs will be used to specifically label wall and determine the relationship between cell wall and outer membrane. Fatty acid auxotrophs will be used to determine the effect of altering fatty acid composition on the synthesis, structure, and function of the outer membrane.

Honors and Awards: None

Publications:

Kulpa, C. F., Jr., and Leive, L.: Mode of insertion of lipopolysaccharide into the outer membrane of *Escherichia coli*. In Fox, C. F. (Ed.): *Biological Membranes - Proceedings of the 1972 ICN-UCLA Symposium in Molecular Biology*. New York, Academic Press, 1972, in press.

Leive, L., and Morrison, D. C.: Preparation of lipopolysaccharides from *Escherichia coli* and other coliform bacteria. In Ginsburg, V. (Ed.): *Methods in Enzymology - Complex Carbohydrates*. New York, Academic Press, 1973, in press.

Serial No. NIAMD-LBP-10
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Chemistry and Mechanism of Pyridoxal Phosphate Enzymes

Previous Serial Number: Same

Principal Investigator: Dr. Edith Wilson Miles

Other Investigator: Dr. Hidehiko Kumagai

Cooperating Units: None

Man Years:

Total:	2.0
Professional:	1.8
Other:	0.2

Project Description:

Objectives:

To gain further understanding of features of enzymes which contribute to their conformation, subunit interaction, and mechanism of action. Pyridoxal phosphate enzymes have been chosen for study because they are important in amino acid metabolism and because pyridoxal phosphate has optical and chemical properties which make it a useful probe of the active site of the enzymes to which it is bound.

Methods Employed:

Chemical modification studies are carried out on a highly purified pyridoxal phosphate enzyme, the B protein of *Escherichia coli* tryptophan synthetase, to determine the presence and role of specific functional groups at the active site and subunit combining site. Optical studies are carried out on the native and modified enzymes to determine changes in the environment of the chromophoric coenzyme, pyridoxal phosphate, and aromatic amino acids in the presence and absence of substrates. The optical methods used include absorbance, fluorescence, circular dichroism, and optical rotatory dispersion spectroscopy. Kinetic studies are conducted to determine the effects of specific modification on subunit interactions, cofactor and substrate binding, and reaction specificities. Pyridoxal amino acid derivatives are isolated

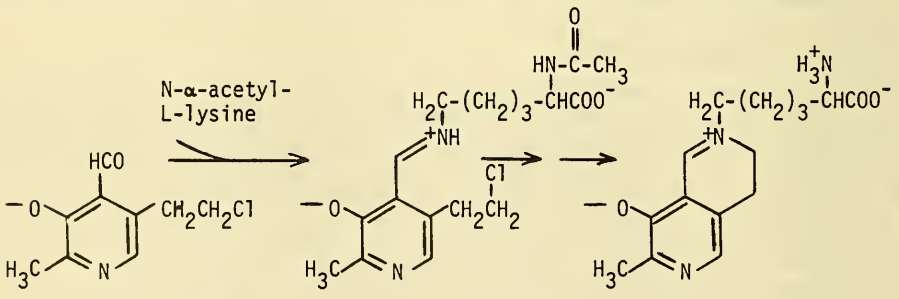
from enzyme hydrolysates or synthetic reaction mixtures by techniques of column chromatography, thin layer chromatography, electrophoresis, and amino acid analysis. The structures of these derivatives are determined by mass spectroscopy and by nuclear magnetic resonance, absorbance, and fluorescence spectroscopy.

Major Findings:

The active site chemistry and mechanism of the B protein of *E. coli* tryptophan synthetase are being studied. The cofactor, pyridoxal phosphate, serves as an optical probe in studies of the enzyme and its enzyme-substrate intermediates by absorbance, fluorescence, and circular dichroism spectroscopy. The effects of chemical modification on these optical properties are observed.

Studies on chemical modification of histidyl residues of the B protein with diethylpyrocarbonate show that one or two histidyl residues are essential for catalytic activity and may function by abstracting the α-hydrogen of the substrate. Modified B protein forms nearly normal complexes with its cofactor (pyridoxal phosphate) and its substrate (L-serine).

A cofactor analog synthesized in this laboratory, α-pyridoxal methylchloride, reacts irreversibly at the active site of the B protein and gives a brightly colored enzyme-derivative which may be a useful indicator at the active site. The product of this reaction has been isolated from acid hydrolysates of the enzyme and shown to be identical to a compound Y synthesized from α^5-pyridoxal methylchloride and N-α-acetyl-L-lysine. The structures of compound Y and its sodium borohydride reduction product have been identified by chemical ionization mass spectrometry and by absorbance and nuclear magnetic resonance spectroscopy. Compound Y is a new cyclic imino acid derivative of pyridoxal and is formed by an intramolecular alkylation by the methylchloride side chain of the Schiff base formed between the ε amino group of lysine and the carbonyl group of α-pyridoxal methylchloride (see Scheme I). This new reaction should be useful for the synthesis of other model cyclic imino acid derivatives of pyridoxal. These cyclic imino acid derivatives are useful as models of imino acid derivatives of pyridoxal and of pyridoxal phosphate



α^5-pyridoxal methylchloride

Y

Scheme I

enzymes since the orientation of the C=N- bond is fixed and the number of possible tautomeric forms is reduced in the cyclic compounds. Initial studies of the absorbance and fluorescence spectra of compound Y as a function of pH have provided valuable information about spectral properties of pyridoxal derivatives.

Significance to Biomedical Research and the Program of the Institute:

These studies are clarifying the active site chemistry and mechanism of pyridoxal phosphate enzymes which are important in amino acid metabolism.

Proposed Course:

These studies will be continued and extended with long range goals of determining chemical and physical features of pyridoxal phosphate enzymes required for specificity, catalytic activity, maintenance of conformation, and protein-protein interaction.

Honors and Awards: None

Publications:

Kumagai, H., and Miles, E. W.: The B protein of *Escherichia coli* tryptophan synthetase. II. New β-elimination and β-replacement reactions. *Biochem. Biophys. Res. Commun.* 44: 1271-1278, 1971.

Miles, E. W.: α-Methylserine transhydroxymethylase (*Pseudomonas*). In Tabor, H., and Tabor, C. W. (Eds.): *Methods in Enzymology*, Vol. 17, Part B. New York, Academic Press, 1971, pp. 341-346.

Serial No. NIAMD-LBP-11
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Molecular Basis of Biologic Specificity

Previous Serial Number: Same

Principal Investigator: Dr. Leonard D. Kohn

Other Investigators: Dr. John M. Utting
Dr. Syed M. Amir

Cooperating Units: (1) Mr. George Poy, Arthritis and Rheumatism Branch, NIAMD, on Amino Acid Analyses
(2) Dr. Roger J. Winand, Hopital de Baviere, Institut de Medecine, Liege, Belgium
(3) Dr. C. M. Lapiere, Department of Dermatology, Hopital de Baviere, Institut de Medecine, Liege, Belgium
(4) Dr. Ralph J. Nossal, Physical Sciences Laboratory, Division of Computer Research and Technology
(5) Dr. S. Kerwar, Roche Institute of Molecular Biology, Nutley, New Jersey
(6) Dr. R. Kaback, Roche Institute of Molecular Biology, Nutley, New Jersey

Man Years:

Total:	2.1
Professional:	2.0
Other:	0.1

Project Description:

Objectives:

Analyses of the molecular bases for enzyme activity and specificity and the mechanisms by which such factors are regulated or determined. Studies of abnormal hormonal factors, their production, and their enzymatic expression.

Methods Employed:

Procedures common to the purification, assay, and chemical modification of enzymes have been coupled with methods evaluating the size, shape,

composition, and sequence of macromolecules.

Major Findings:

Relationships of Thyrotropin and Exophthalmogenic Factor -- Previous work demonstrated that TSH was exophthalmogenic but that partial pepsin digestion of TSH could yield an exophthalmogenic fragment of the TSH molecule which had lost its thyroid stimulating ability. The structure of this fragment has now been carefully characterized; it consists of all of the β subunit and an amino terminal fragment of the α subunit of TSH. This fragment and TSH cause exophthalmos in fish, guinea pigs, and mice. The exophthalmos is associated with an increased synthesis and accumulation of glycosaminoglycans of which hyaluronic acid is the major changing component. Sulfation of glycosaminoglycans parallels the changes in hyaluronic acid and is preceded by SO_4 transport and intracellular activation to PAPS (phosphoadenosine phosphosulfate). TSH and the exophthalmogenic fragment bind to the plasma membranes of guinea pig retroorbital tissue and the binding is associated with adenylate cyclase activation. Gamma globulin from patients with exophthalmos significantly affect binding; this gamma globulin is not LATS (the long acting thyroid stimulating gamma globulin). A mechanism for human exophthalmos has been proposed as a consequence of these data. Experiments designed to test this hypothesis have been initiated.

Procollagen and Procollagen Peptidase -- Cattle with a recessive anomoly, dermatosparaxis, have been studied. These animals with dramatic skin changes have been shown to accumulate precursor forms of the collagen α_1 and α_2 chains. These precursor forms are larger than the normal α_1 and α_2 chains, the increased size being caused by a peptide extension at their amino termini. Dermatosparaxis has been shown to be caused by a missing enzyme, procollagen peptidase, which cleaves pro α_1 and pro α_2 to yield α_1 and α_2, respectively. The enzyme has been characterized in normal cattle tissues and shown to be elevated in activity in several human disease states. A relationship of procollagen and procollagen peptidase to aging has been postulated. The procollagen chains have been synthesized with an *in vitro* polysome system in a 2 : 1 ratio, *i.e.*, their precursor nature has been definitively established. This system is stimulated by initiation factors.

Structure-Function Studies of Hydroxypyruvate Reductases -- A hydroxypyruvate reductase has been induced in *P. acidovorans* by growth on glyoxylate. After purification to homogeneity, as shown by ultracentrifugation and disc gel electrophoresis, the enzyme is stable in 0.04 M Tris Cl, pH 7.4, containing 25% glycerol and 0.1 M NaCl; it has an $s^{o}_{25_w}$ of 4.5 S to 5.0 S and a molecular weight of approximately 80,000. The constitutive enzyme, also present in the glyoxylate-grown organism, has values of 4.8 S and 75,000, respectively [*J. Biol. Chem.*, 243, 2492 (1968)]. From SDS gels a subunit molecular weight of 40,000 has been calculated for the induced enzyme. The induced enzyme has a different amino acid composition, absorption spectrum, and isoelectric point. It has a higher pH optimum, is competitively inhibited by glyoxylate, and has an inversed affinity for reduced pyridine nucleotides, *i.e.*, it has a higher "K_m" for DPNH than TPNH. Both enzymes are specific for hydroxypyruvate; however, in the induced enzyme, the reaction is more readily reversed by DPNH and D(-)-glycerate. The induced enzyme is inhibited by SO_4, Cl,

and NO_3; PO_4 exhibits activation and then inhibition, *i.e.*, negative cooperativity. The constitutive enzyme is only activated by these anions. The subunits of the induced enzyme are identical and can reassociate to yield a protein having the same physical and kinetic properties as native enzyme. The yield of reassociation is nearly 100%, the half-time is less than 2 minutes, and the kinetics are compatible with a simple bimolecular reaction. Preliminary evidence indicates that the induced enzyme is a membrane enzyme, whereas the constitutive is cytoplasmic in location.

The amino acid sequence of an active site peptide from glyoxylate reductase has been determined. A striking similarity exists with the sequence of an analogous peptide in glyceraldehyde 3-phosphate dehydrogenase.

Bacterial Chemotaxis -- Studies have been initiated to determine force-response relationships pertaining to chemotactic motions of *E. coli* K_{12} bacteria. Assay techniques for bacterial motion utilize newly developed methods of laser light intensity correlation spectroscopy. The isolation of chemotactic receptors is also being attempted, with subsequent analysis of receptor function when embedded in reconstituted lipid bilayer membranes.

Membrane Transport -- D-Lactate has been demonstrated to stimulate bacterial transport of amino acids and sugars. This enzyme has been partially purified from *E. coli* and shown to be nonsensitive to PCMB, NEM, and other SH inhibitors, whereas these same reagents significantly inhibit bacterial transport. The enzyme can exist in large molecular weight complexes with cytochrome b_3. It contains bound flavin in a form important to enzyme function and is not DPNH or DPN dependent.

Thyrotropin Activation of the Thyroid -- Thyroid membranes have been prepared and characterized. Specific binding of TSH and its subunits has been demonstrated. A new binding assay has been developed which shows that binding is immediate and precedes adenylate cyclase activation.

Significance to Biomedical Research and the Program of the Institute:

Analyses of the molecular bases for activity and specificity and the mechanisms by which these determinants are influenced offers a further understanding of the nature of metabolic and endocrine defects. The possibility that unregulated biosynthetic or degradative processes may convert TSH into EPS active material has been established, and a mechanism for human exophthalmos has been proposed on the bases of these data. Preliminary studies of procollagen, procollagen peptidase, and collagen biosynthesis suggest an important relationship to aging.

Proposed Course:

Structure-function analyses of the reductase model system are being pursued. The localization of the active and regulatory sites on each enzyme will be sought and the comparative structures of these units will be compared. Comparison of membrane bound and free enzymes will be made. Similar studies of the TSH-exophthalmic factor relationship will be continued in an attempt to examine the mechanism by which EPS activity is generated *in vitro* and *in vivo*.

The proposed mechanism for human exophthalmos will be tested.

Honors and Awards:

Visiting Professor, University of Liege, Liege, Belgium

Publications:

Kohn, L. D., and Winand, R. J.: Relationship of thyrotropin to exophthalmos-producing substance. Formation of an exophthalmos-producing substance by pepsin digestion of pituitary glycoproteins containing both thyrotropic and exophthalmogenic activity. *J. Biol. Chem.* 246: 6570-6575, 1971.

Lapiere, C. M., Lenaers, A., and Kohn, L. D.: Procollagen peptidase: an enzyme excising the coordination peptides of procollagen. *Proc. Nat. Acad. Sci. U.S.A.* 68: 3054-3058, 1971.

Nossal, R. J.: The growth and movement of rings of chemotactic bacteria. *Exp. Cell Res.*, in press.

Winand, R. J., and Kohn, L. D.: The binding of [^{3}H]-thyrotropin and an [^{3}H]-exophthalmogenic factor by plasma membranes of retroorbital tissues. *Proc. Nat. Acad. Sci. U.S.A.*, in press.

Serial No. NIAMD-LBP-12
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Role of Subunit Interactions in Enzyme Chemistry

Previous Serial Number: Same

Principal Investigator: Dr. Claude B. Klee

Other Investigators: None

Cooperating Units: Dr. Werner A. Klee, Laboratory of General and Comparative Biochemistry, National Institute of Mental Health
Drs. Louis A. Cohen, Kenneth L. Kirk, and Herman J. C. Yeh, Laboratory of Chemistry, NIAMD
Dr. Jack S. Cohen, Division of Computer Research and Technology

Man Years:

Total:	1.2
Professional:	1.0
Others:	0.2

Project Description:

Objectives:

Two enzymatic systems, histidine ammonia-lyase and lactose synthetase, are used to study the possible regulatory functions of protein-protein interactions.

Methods Employed:

The project involves the study of properties of proteins, purified by the usual techniques of protein isolation, including many types of chromatography. The enzymes are being studied by examining their optical, hydrodynamic, and kinetic properties as well as by suitable chemical measurements and modifications.

Major Findings:

Histidine Ammonia-Lyase -- Active Site Structural Studies. Histidine ammonia-lyase has previously been shown to be greatly stimulated by some metal

ions. Four reactive cysteinyl residues (per mole of tetrameric enzyme) provide at least part of the high affinity sites for these metal ions. Thus these cysteinyl residues can be considered to be at the active site of the enzyme. In collaboration with Dr. J. A. Gladner of the Laboratory of Biophysical Chemistry, NIAMD, we have isolated and characterized a single peptide containing this cysteinyl residue in a yield of four moles per mole of enzyme. This result is a confirmation of the tetrameric structure of the enzyme. The active site has also been shown to contain a dehydroalanyl residue (Dr. R. B. Wickner) which is thought to interact with the amino group of the substrate. I am now trying to isolate a peptide containing this moiety to further document the nature and the number of active sites.

Treatment of the enzyme with L-cysteine in the presence of oxygen results in an irreversible inactivation and in the incorporation of 3-3.5 moles of cysteine per mole of enzyme. This modification seems to affect that part of the protein containing the dehydroalanyl residue since the inactivated enzyme no longer yields tritiated alanine after reduction with tritiated $NaBH_4$. Furthermore, the reaction with cysteine is stereospecific; D-cysteine is inactive. Since L-cysteine and not D-cysteine is a competitive inhibitor of the enzyme, this type of modification seems to be an active site specific modification. Preliminary studies on the nature of the reaction indicates that cysteine is incorporated neither as cystine nor as lanthionine. When [^{14}C]cysteine is used, the radioactivity is recovered from the amino acid analyzer as a single component after acid hydrolysis. I am now trying to identify this derivative and also to isolate a tryptic peptide containing this residue.

Mechanism of Action. Using an analog of histidine, 4-fluoro-L-histidine, synthesized by Drs. K. L. Kirk and L. A. Cohen of this Institute, we have tried to obtain more information on the mechanism of action of the enzyme. The following sequence of reactions has been proposed previously by Dr. A. Peterkofsky:

$$\text{histidine} + \text{enzyme} \rightleftarrows \text{urocanic acid} + H^+ + \text{amino-enzyme} \quad (1)$$

$$H^+ + \text{amino-enzyme} \rightarrow \text{enzyme} + NH_3 \quad (2)$$

We have now shown that 4-fluoro-L-histidine, which is a strong competitive inhibitor of the enzyme (K_i : 10^{-3} M) is a very poor substrate. The turnover number is 1/200th that of L-histidine. Since the K_m is the same as that of L-histidine and since reaction (2) must be the same for both substrates, the rate limiting step in the deamination of fluoro-histidine has to be the formation of fluoro-urocanate [reaction (1)]. Furthermore, the deamination of both substrates is stimulated to an equal extent by metal ions and it is therefore reasonable to assume that this step is also rate limiting in the case of L-histidine. Indeed, when the initial rate of the reaction was measured with stoichiometric amounts of enzyme, we failed to detect any "burst" of activity as would have been the case if reaction (2) was the rate limiting step. Preliminary experiments performed with stopped flow rapid kinetic techniques indicates that after an initial lag the initial rate is indistinguishable from the steady state rate.

A more direct and detailed study of the mechanism of action of the enzyme is now being pursued in collaboration with Drs. L. A. Cohen, K. L. Kirk,

H. J. C. Yeh, and J. S. Cohen, using NMR techniques. The presence of the fluorine atom on the imidazole ring of the substrate will enable us to follow the chemical shifts of this atom upon addition of enzyme and metal ions and will allow us to test the hypothesis that the metal stimulation is due to an interaction of the metal ions with the imidazole ring resulting in a labilization of the β hydrogen.

Lactose Synthetase. Studies with lactose synthetase have been concentrated on the properties of the membrane bound form of the A protein. In rat brain the enzyme is found primarily in the synaptosomal fraction and it may readily be solubilized from this source with detergents and purified by affinity chromatography on columns of α lactalbumin bound to Sepharose. The kinetic properties of this partially purified enzyme are indistinguishable from that of the pure A protein previously obtained from milk.

Significance to Biomedical Research and the Program of the Institute:

These two oligomeric systems, histidine ammonia-lyase composed of four identical subunits and lactose synthetase, a complex of two different proteins, are being studied to elucidate the role of protein-protein interactions in the mechanism of their catalytic function. It is expected that they will complement one another in our effort to understand the physiological significance of macromolecular interactions.

Proposed Course:

Future work with histidase will continue to be aimed at the elucidation of the structure of the active site which will enable us to understand the mechanism of action of this enzyme. Ultimately, we hope to try to determine the role which intersubunit interactions play in the enzyme mechanism. Further studies with lactose synthetase will be carried out to try to elucidate the role of this enzyme in membrane synthesis and function.

Honors and Awards: None

Publications:

Klee, C. B.: Histidine ammonia-lyase (*Pseudomonas*). In Tabor, H., and Tabor, C. W. (Eds.): *Methods in Enzymology*, Volume 17, Part B. New York, Academic Press, 1971, pp. 69-73.

Klee, C. B.: Primer-dependent polynucleotide phosphorylase. In Cantoni, G. L., and Davies, D. R. (Eds.): *Procedures in Nucleic Acid Research*, Volume 2. New York, Harper and Row, 1971, pp. 896-911.

Klee, C. B.: Metal activation of histidine ammonia-lyase: metal ion-sulfhydryl group relationship. *J. Biol. Chem.* 247: 1398-1406, 1972.

Klee, W. A., and Klee, C. B.: The interaction of α-lactalbumin and the A protein of lactose synthetase. *J. Biol. Chem.* 247: 2336-2344, 1972.

By the time of the next Annual Report LPB will have undergone fission, and the name, along with about half our members, will have moved out of quarters in Building 2, occupied for 35 years, into renovated space in Building 6. The colleagues remaining in Building 2 will constitute the new Laboratory of Chemical Physics, under Dr. E. D. Becker. Thus the Laboratory of Industrial Hygiene, which, as the Hygienic Laboratory (1891) was the original research arm of the U. S. Public Health Service and was the first Laboratory on the NIH Bethesda campus (1938), will have at least four organizational descendents: (a) the Laboratory of Physical Biology, NIAMD (1947), (b) the Laboratory of Physiology, NCI (originally the Radiation Branch) (1953), (c) the Laboratory of Biophysical Chemistry, NIAMD (1963) and (d) the Laboratory of Chemical Physics, NIAMD (1972).

The scientific and social evolution of Industrial Hygiene in terms of people and projects is hard to trace through the comings, goings, growth and attrition of nearly four decades, but old timers will remember (1) the Lab Chief genealogy of R. R. Sayers, J. G. Townsend, P. A. Neal and Heinz Specht, (2) Albert Szent-Gyorgyi, R. W. G. Wyckoff, F. S. Brackett, Leon Heppel and Bernard Horecker among distinguished alumni, (3) the frenetic days of aviation medicine and DDT pharmacology during World War II, (4) playing noon softball on the site of the Clinical Center and tending victory gardens on the Building 31 parking lot, (5) working under the banner of the Experimental Biology and Medicine Institute, before NIAMD existed, and (6) the advent of big money, the institution of intramural postdoctoral fellowships and the impacts of electron microscopy, chromatography, enzymology and xerography, among other landmarks of NIH history.

Taking 1947 as the start of the golden age of biomedical research, and confining attention only to the pure LPBers of Building 2, it may be of interest to estimate the personnel flow and productivity record of the past 25 years. In 1947 the Laboratory had about 95 bodies on board, and in 1972 the total is 59 (including 17 temporaries), of whom only 9 date back to 1947 or earlier. The 50 newcomers represent the result of filtering over two hundred birds of passage who came to us by civil service transfer and outside recruitment. Steady state was maintained by transfer (65), retirement (11), voluntary or involuntary travel to greener pastures (44), return to home institutions (35) and death in harness (7). Our transients have included 16 young medicos being blooded in bench work, 20 Staff Fellows and 38 foreign scientists who have spent one or more years with us, their countries of origin being Japan (13), Australia (4), Israel (4), 2 each from Great Britain, Italy, India and Norway, and 1 each from Canada, Chile, France, Germany, Holland, Mexico, Pakistan, the Philippines and Taiwan. To total the papers published since LPB became an entity would demand much antiquarian research, but the sum listed in the last 10 Annual Reports is 407, aggregating 3,212 pages.

As may be imagined, the impending schism has engendered physical as well as psychic trauma, so it is appropritate, in closing, to call attention to the Cash Award recently given Howard Brubach for his role in easing the pangs of motion for both the migrants and the spreading remainders. Needless

to say Mr. Brubach, as our most senior member, was a tower of strength also in the compilation of this nostalgic recital.

Spectroscopy

Levin and coworkers developed apparatus for the study of Raman spectra at liquid helium temperatures and utilized it in the investigation of matrix-isolated CCl_4 and of acetone and acetone-d_6. Features not usually observable at room temperature can be discerned under these conditions and aid in the detailed interpretation of the spectra.

McDiarmid's study of the electronic changes occurring in simple chromophores upon absorption of UV photons has led to an assignment of most of the spectral bands observed in diethyl ether. She has also shown that the long accepted interpretation of the spectrum of liquid ethylene was erroneous because of the presence of atmospheric oxygen in the sample.

Kon reported the first observation by electron spin resonance of the triplet ground state of molecular oxygen in a condensed phase--a matrix of N_2 or CO at $\leq 10°$ K. He and his coworkers also used ESR to examine the bonding in a copper-doped histidine hydrochloride crystal and the complexing of species-varying hemoglobins with haptoglobin.

Becker and coworkers used ^{23}Na NMR to study the state of sodium in blood and muscle cells, concluding that the red cell has no sequestered or non-exchangeable solute pools. They employed double resonance methods to elucidate the signs of various coupling constants in ^{15}N-enriched oxaziridines, and a combination of NMR, IR and vapor pressure methods was applied to an analysis of the hydrogen bonded species present in t-butanol dissolved in a hydrocarbon solvent.

Macromolecular Behavior and Properties

The electric field-induced dichroism in DNA and synthetic polynucleotides has been studied by Charney and coworkers. Their findings permit the assignment of electronic transitions in poly-A and the elucidation of subtle details of its molecular conformation in solution.

Eaton has used polarized single crystal absorption spectra to assign a number of electronic transitions in heme proteins and to examine very subtle conformational changes in these molecules. Eaton has also investigated several non-heme iron-sulfur proteins by single crystal spectrophotometry and by infrared circular dichroism.

By electron microscopy Labaw has shown that the satellite tobacco necrosis virus consists of a central sphere of nucleic acid about 108 Å in diameter surrounded by protein subunits probably having icosohedral symmetry.

Murayama showed that the effect of low concentrations of urea in preventing or reversing the polymerization of sickle cell hemoglobin (and the consequent distorting of the erythrocytes) is on the ordered water layer between adjacent hemoglobin molecules.

Physical Organic Chemistry

Weiss and coworkers completed the structural elucidation of several pigments from certain plants and from certain aphids. Surprisingly, naphthoquinone methides have been found in both types of sample, and in addition the aphid pigments contain novel forms of carotene.

Ziffer and coworkers analyzed circular dichroism data for several dihydroxydienes in terms of absolute configuration, conformational equilibria and the presence of previously undetected absorption bands. Charney, Weiss and Ziffer found and interpreted an inversion of the previously expected sign of the chiral-optical effects in non-planar heteroannular cisoid dienes.

Adams, Sharpless and Jennings found that the Schiff's base formed between phosphatidyl ethanolamine and retinaldehyde undergoes a change in absorption spectrum from 340 nm to 365 nm on interaction with water. These experimental studies, together with Hückel molecular orbital calculations for retinaldehyde and related compounds, lend support to the hypothesis that an intramolecular π complex of the Schiff's base may be the visual pigment.

Membrane and Monolayer Physical Chemistry

Gershfeld showed that there is association between cholesterol and the polar moieties of phospholipids. Contrary to current concepts, the acyl chains of the phospholipid do not enhance the association with cholesterol, but may actually reduce the interaction energy.

Sollner and Shean, using strong base liquid anion and cation exchanger membranes, have extended their measurements of bi-ionic potentials and poly-ionic potentials, obtaining excellent agreement with theoretical predictions. Salts of dipicrylamine dissolved in high dielectric constant solvents proved excellent strong acid cation exchanger liquid membranes for use in studies on bi-ionic potentials.

Studying the effects of electrolytes on the phospholipid bilayers formed in aqueous lecithin gels, Gottlieb found that neither the calorimetrically measured heats nor the temperatures of a crystalline to liquid crystalline transition undergone by the bilayers were affected by Na_2SO_4, $MgCl_2$ and $Ca\ Cl_2$. Incorporation of small amounts of a second lipid, hexadecanol, into the lecithin bilayers markedly increased both the heat and the temperature of the transition.

Biochemistry

F. Hagins, with the cooperation of Levenbook, found that highly purified preparations of squid rhodopsin possess an N-terminal glycine residue, consist of single peptide chains of molecular weights 50,000 ± 1,000 and contain some carbohydrate.

Levenbook and Bauer reported that the small peptides in Calliphora fly blood are cleaved by peptidase(s) in inverse proportion to their concentrations.

Levenbook and Sridhara found that during the metaphorphosis of the fly Calliphora 40% of previously intracellular ribosomes become extracellular and are presumably destroyed.

Kempner found no free sugars in the pool of metabolic intermediates in Euglena gracilis. The predominant compound is D-mannitol and the two biochemical pathways of mannitol known in bacteria are both present in Euglena.

By sucrose gradient differential centrifugation and electron microscopy Hopkins and Hanna have isolated the "photocyte granule" of the firefly light organ, identified it with the peroxisome (microbody) of many vertebrate tissues and shown that it contains catalase and the bioluminescent system.

Excitable Physiological Systems

Studying skinned frog muscle fibers, Podolsky and associates have found that (a) the sarcoplasmic reticulum takes up calcium fast enough to account for the physiological relaxation rate, (b) the calcium uptake process is accelerated by magnesium. The catalytically active species is Mg^{2-}, and (c) the force developed by the cross-bridges in muscle cells does not depend on the regularity of the lattice formed by the myofilaments.

Hagins, Yoshikami and Rüppel have discovered a new light-induced ionic current, the "L" current, in the envelope membranes of rat retinal rod outer segments in solutions containing very low concentrations of Ca^{++} ions. This current is somewhat slower than the decay of metarhodopsin I but precedes the electrical events now believed to signal neural excitation in rods. The "L" current is suppressed by light adaptation of rods and by Ca^{++} applied externally to them. Thus one more piece of evidence is provided showing the similarity of the action of Ca^{++} and light on rods and implicating Ca^{++} as an internal excitatory transmitter in rods and cones.

Extending the work reported last year on the entrainment of the New Guinea firefly genus Pteroptyx to rhythmic flashes of electric light, J. and E. Buck have found that the closely similar genus Luciola uses the relaxation oscillator-reset mechanism of phase-shifting only with pacers very close to the animals' free-run period; with more divergent pacers, potentially out-of-phase flashes are inhibited entirely.

Stress Physiology

Altland and associates have shown that polycythemia induced by hypoxia or cobalt in rats does not adversely affect performance of moderate exercise unless tissue changes such as renal and hepatic lipoidosis and focal myocardial necrosis are also present. A negative cross adaptation has been found to exist between cold-acclimation and hypoxia administered at room temperature. This appears to be principally the result of the persistance of an elevated metabolic rate after removal from cold.

Serial No. NIAMD-LPB-1
1. Physical Biology
2. Cellular Physics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Mechanism of Muscular Contraction

Previous Serial Number: Same

Principal Investigator: R. J. Podolsky

Other Investigators: Mark Schoenberg
A. C. Nolan
Marc D. Thames
Elizabeth W. Stephenson

Man Years

Total:	7
Professional:	6
Other:	1

Project Description:

Objectives:

1. To work out the molecular mechanism of muscular contraction.
2. To understand the control processes for contractility.

Methods Employed:

1. Analysis of the motion and the X-ray diffraction pattern of both intact muscle fibers and "skinned" fiber segments under chemically controlled conditions.
2. Study of the electrical and chemical parameters involved in the activation of various types of muscle fibers.
3. Measurement of ionic fluxes across the internal membranes of skinned muscle fibers under various conditions.

Major Findings:

1. Movements of calcium from the sarcoplasmic reticulum into the myofilament space (which activates muscle contraction) appears to be triggered by the entry of a small amount of extracellular calcium into the myofilament space.

2. The sarcoplasmic reticulum takes up calcium fast enough to account for the physiological relaxation rate.
3. The calcium uptake process is accelerated by magnesium. The catalytically active species is Mg^{2+} rather than $MgATP^{2-}$.
4. The force developed by the cross-bridges in muscle cells does not depend on the regularity of the lattice formed by the myofilaments.
5. The time course of force development in the first few milliseconds after a length step (Huxley & Simmons, Nature, 233, 533 (1971)) is well described by the cross-bridge mechanism put forward by this laboratory (Podolsky et al., PNAS, 64, 504 (1969)).

Significance to NIAMD Research:

The elucidation of the molecular mechanism of muscular contraction together with the chemistry of the activation process, can be useful in the rational handling of neuromuscular and cardiovascular disease.

Proposed Course of Project:

1. The influence of various agents (pCa, pH, ionic strength, metal ions, ATP) on the ability of muscle fibers to develop force and to shorten will be examined directly in skinned fiber segments.
2. The influence of these same agents on myofilament spacing and the cross-bridge configuration will be examined in skinned fibers by X-ray diffraction.
3. The mechanical response of muscle fibers to controlled changes in load will be compared with the motion predicted from a cross link contraction model with previously characterized properties.
4. Movement of calcium between the myofilament space and the sarcoplasmic reticulum will be studied by tracer methods.

Publications:

Ford, L. E., and Podolsky, R. J.: Calcium uptake and force development by skinned muscle fibers in EGTA buffered solutions. J. Physiol., in press.

Ford, L. E., and Podolsky, R. J.: Intracellular calcium movements in skinned muscle fibers. J. Physiol., in press.

Schoenberg, M., and Podolsky, R. J.: Length-force relation of calcium activated muscle fibers. Science, in press.

REPORTED IN PRESS - 1971

Editor: Podolsky, R. J.: Contractility of Muscle Cells and Related Processes. Englewood Cliffs, New Jersey, Prentice-Hall, 1971.

Podolsky, R. J., and Nolan, A. C.: Cross-bridge properties derived from physiological studies of muscle fibers. In Podolsky, R. J. (Ed.): Contractility of Muscle Cells and Related Processes. Englewood Cliffs, N. J., Prentice-Hall, 1971, pp. 247-260.

Serial No. NIAMD-LPB-2
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Triggering of Bioluminescence. Sense of Rhythm.

Previous Serial Number: NIAMD-LPB-2

Principal Investigator: John B. Buck

Other Investigators: Dr. Thomas A. Hopkins; Mr. Charles H. Hanna

Cooperating Units: Mrs. Elisabeth M. Buck, Guest Worker; Dr. Frank A. Hanson, University of Maryland, UMBC, Catonsville, Maryland; Dr. James F. Case, University of California, Santa Barbara, California.

Man Years:

Total:	5.0
Professional:	3.0
Other:	2.0

Project Description:

Objectives:

To understand how action potentials in nerves are coupled to chemical light production in luminous organs. To understand how individual central nervous oscillators can become mutually entrained.

Methods Employed:

Standard biochemical and spectroscopic; pharmacological; psychophysical: electron microscopy.

Major Findings:

1. Starting from the electron microscopical similarity of firefly photocyte granules and the peroxisomes of various vertebrate tissues Hopkins and Hanna have demonstrated catalase activity in these intracellular particulates isolated by sucrose gradient differential centrifugation. The granules were also shown to be the containers of the bioluminescent system.

2. Extending the work reported last year on the entrainment of the New Guinea firefly genus Pteroptyx to rhythmic flashes of electric light it has now been found that the closely similar genus Luciola does not use the relaxation oscillator-reset mechanism of phase-shifting. With pacers very close to the animal's free run period the period can be lengthened or shortened slightly to maintain minimal phase difference; with more divergent pacers potentially out-of-phase flashes are inhibited entirely (J. Buck, E. Buck and F. Hanson).

Significance to NIAMD Research:

The new view of peroxisome function in the light organ may permit a better understanding of how intracellular luminescence is controlled. The new findings on Luciola entrainment illustrate the astonishing plasticity of the insect nervous system in accomplishing a particular objective (synchronization) by several quite different approaches.

Proposed Course of Project:

No major changes anticipated.

Honors:

National Research Council -- three year term.

Publications:

Hanson, Frank E., Case, James F., Buck, Elisabeth, and Buck, John: Synchrony and flash entrainment in a New Guinea firefly. Science 174: 161-164, 1971.

White, Emil H., Rapaport, Eliezer, Seliger, Howard H., and Hopkins, Thomas A.: The chemi- and bioluminescence of firefly luciferin: An efficient chemical production of electronically excited states. Bioorganic Chem. 1: 92-122, 1971.

REPORTED IN PRESS - 1971

McLean, Miriam, Buck, John, and Hanson, Frank E.: Culture and larval behavior of photurid fireflies. Amer. Midland Naturalist 87: 133-145, 1972.

Serial No. NIAMD-LPB-3
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Biochemistry of Blowflies.

Previous Serial Number: NIAMD-LPB-3

Principal Investigator: L. Levenbook

Other Investigators: A. Bauer and S. Sridhara

Cooperating Units: F. Hagins (NIAMD-LPB-11); J. Collet (University of Sussex, England)

Man Years

Total:	2-1/2
Professional:	2-1/2
Other:	0

Project Description:

Objectives:

1. To characterize and compare purified muscle and fat body aldolases from larval and adult blowflies.

Methods Employed:

Routine micro-chemical procedures for peptide isolation and identification; routine physical methods for isolation of fly ribosomes and standard radiochemical procedures.

Major Findings:

1. Firefly peptides. Larvae, pupae and adult fireflies all contain sizeable pools of free amino acids, albeit quantitatively less than Diptera and Lepidoptera. Of particular interest were 5-6 unknown peaks on the amino acid analyzer which turned out to be peptides. To date, only one of these has been unequivocally identified as ß-aspartyl-histidine. This peptide also occurs in high concentration in the developing adult light organ.

2. Calliphora peptides. Adult Calliphora contain a wide range of small peptides, but the total amount may vary widely. The fly possesses active peptidase(s) that cleaves the endogenous peptides, at least

in vitro. An inverse correlation has been demonstrated between the rate at which the peptidase(s) cleave various peptides, and their abundance in the insect; most slowly attacked peptides seem to be the most abundant. The peptidase(s) is inhibited by both high concentration of substrate, and by free amino acids. It is proposed that peptidase activity is controlled by either (or both) free peptides and/or amino acids.

3. Aldolase. While all work over the last few years indicates that larval and adult Blowfly muscle aldolases are identical, it now appears that the corresponding fat-body enzymes are different. This conclusion (if correct) is philosophically perplexing, and the fat-body work is in process of being repeated. Amino acid composition and peptide mapping of the muscle enzymes are now in progress.

4. *Calliphora* ribosomes. Progress on *Calliphora* ribosomes can be summarized as follows: (a) during metamorphosis, some 40% of the previously intracellular ribosomes become extra-cellular and are presumably destroyed, since a marked reduction in total ribosomes occur during the larval-adult transition. Further, during adult-development, differences are observed in the ratio of (intracellular membrane bound) to (intracellular-free) ribosomes.

Significance to NIAMD Research:

Aside from glutathione and peptide hormones, the function of ubiquitous small peptides in nature is largely unknown; insects, therefore, are the material of choice for this type of study. Likewise, the fate of intracellular ribosomes during drastic tissue breakdown and histogenisis can most conveniently be examined during insect metamorphosis. The structure and function of a major protein component in an invertebrate forms a useful model system for mammalian protein biochemistry.

Proposed Course of Project:

Continuation of insect ribosome project; detailed study of structure and biological function of calliphorin; identification of remaining firefly peptides.

Publications:

Dinamarca, M. L., Levenbook, L. and Valdés, E.: DDT-dehydrochlorinase II. Subunits, sulfhydryl groups, and chemical composition. *Arch. Biochem. Biophys.* 197: 374-383, 1971.

REPORTED IN PRESS - 1971

Levenbook, L., Hutchins, R.F.N., and Bauer, A.C.: Uric acid and basic amino acids during metamorphosis of the tobacco hornworm, *Menduca sexta*, with special reference to the meconium. *J. Insect Physiol.* 17: 1321-1331, 1971.

Serial No. NIAMD-LPB-4
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Molecular Mechanism of Human Red Cell Sickling and Unsickling

Previous Serial Number: NIAMD-LPB-4

Principal Investigator: Makio Murayama

Other Investigators: Dr. Shiro Ohnoki

Cooperating Units: None

Man Years

Total:	2
Professional:	2
Other:	0

Project Description:

Objectives:

1. To find a molecular mechanism of "unsickling" of erythrocytes from sickle cell anemia patients.

2. To evaluate thermodynamic constants: the enthalpy change in the thermal (endothermic) aggregation of sickle cell hemoglobin (Hb S); the entropy change in the endothermic aggregation of Hb S; and the volume of activation in the endothermic aggregation of Hb S.

3. To investigate the mode of action of urea in unsickling.

4. To examine the specificity of the oligodynamic action of urea.

5. To investigate the effect carbamylation of amino 1 of the β-chain by cyanate (or carbamyl phosphate) on sickling.

Methods Employed:

Aggregation-deaggregation of sickle cell hemoglobin by pressure, temperature, and millimolar urea. Structural details of sickling and unsickling by differential interference optical (Nomarski optics) microscopy.

Major Findings:

The volume of activation (ΔV^*) of sickle cell hemoglobin in the thermal (endothermic) aggregation is 400 cc $mole^{-1}$. The enthalpy change (ΔH^*) = the energy of activation = 16 kCal $mole^{-1}$. The entropy change is 52 e. u. which means that 10 water molecules are excelled from between adjacent Hb S molecules so that intertetramic hydophobic interactions will take place. Ten to 20 millimolar urea is adequate to act on the clathrate (the hydration layer about the apolar residues) to break the hydrophobic interactions; this is called the oligodynamic action of urea. Urease completely erases the oligodynamic action of urea even when the therapeutic level of 150 to 170 mg per 100 ml of blood is used. It is shown that the mode of action of potassium cyanate is entirely different from that of urea: urea reverses and blocks the sickling phenomenon; cyanate (or carbamyl phosphate) only blocks the sickling phenomenon. Potassium cyanate cannot, therefore, ever be considered as a therapeutic agent in sickle cell disease.

Significance to NIAMD Research:

Sickle cell anemia is a hereditary, non-infectious hemolytic anemia, hence significant to the NIAMD mission. My theory has been applied at the bed side with success here as well as in Africa (oral urea therapy and oral urea prophylaxis).

Proposed Course of Project:

The molecular mechanism of unsickling of sickled human erythrocytes will be explored further from the standpoint of bioenergetics. It appears that there is a dialyzable small molecule which is the co-factor for Hb S to aggregate. Due to lack of sufficient material the co-factor has not yet been characterized. This project will be pursued.

Honors and Awards:

Institute for Health Living. Wayne State University Award of $1,000, January 20th, 1972, after delivering my "The Individual's Science Lecture".

Martin Luther King Foundation Award for 1972, May 31, 1972, in Philadelphia, Pennsylvania.

Special Travel Award of University of Science and Technology, Kumasi, Ghana, to attend West African Society for Pharmacology. Scientific Meeting 18th - 19th March, 1972.

Honorary Member West African Society for Pharmacology, 3-19-72.

Publications:

REPORTED IN PRESS - 1971

Altland, P. D., Brubach, H. F., Parker, M. G., Dieter, M. P., AND Murayama, M.: Effects of smoke on tolerance of rats to hypoxia. J. Appl. Physiol. 30: 352-357, 1971.

Murayama, M.: The chemical and the three-dimensional structure of human hemoglobin. Annals of Clin. Lab. Sci. 1: 1-9, 1971.

Serial No. NIAMD-LPB-5
1. Physical Biology
2. Electrochemistry and Colloid Physics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Physical Chemistry

Previous Serial Number: NIAMD-LPB-6

Principal Investigator: M. H. Gottlieb

Other Investigators: None

Cooperating Units: Dr. E. D. Eanes, NIDR

Man Years:

Total:	1-1/3
Professional:	1
Other:	1/3

Project Description

Objectives:

To understand the interactions between lipids, water and electrolytes, and the effects of such interactions on structures formed by the lipid.

Methods Employed:

X-ray diffraction, calorimetry.

Major Findings:

Mixtures of the phospholipid lecithin with water have a lamellar structure in which water layers separate bimolecular layers of the lecithin. It is known that the hydrocarbon chains of the latter undergo a sharp transition from a highly crystalline state to a mobile, liquid-crystalline like state at a temperature considerably below the true melting point. A study was made of the effects of various electrolytes on the temperature, T_t, and the heat, q_t, of the transition. Both T_t and q_t were unaffected by the presence of KCl, NaCl, LiCl, Na_2SO_4, $MgCl_2$ and $CaCl_2$. In the presence of HCl, the transition starts at the same temperature as with pure water, but takes place over a temperature range of about 10°C; q_t is the same as for pure water. With $BaCl_2$ and $SrCl_2$, T_t is the same as for pure water, but q_t is significantly smaller. Although the effects of HCl are undoubtedly a result of the association of

H^+ ions with the phosphoryl groups of lecithin, a detailed explanation must await additional, X-ray diffraction, data. The effects of $BaCl_2$ and $SrCl_2$ are seemingly consistent with increased complex formation between the cations and the phosphoryl groups of the lecithin, on going from the crystalline to liquid crystalline states.

Studies were started on the effects of incorporating a second lipid into the lecithin bilayers. Small amounts of hexadecyl alcohol significantly increase both q_t and T_t. In the course of these experiments the interesting observation was made that the crystalline-liquid crystalline transition of pure hexadecyl alcohol is not reversible, i.e. the transition from the liquid crystalline to crystalline states place readily, while the reverse transition does not occur.

Proposed Course of the Project:

The effects, on the crystalline to liquid crystalline transition behavior of phospholipid bilayer,, of incorporating hexadecyl alcohol and other simple lipids into the bilayers will be studied in detail. Parallel X-ray diffraction studies of the structures of the bilayers are also planned. The factors affecting the reversibility of the phase transition with pure hexadecyl alcohol will be investigated.

Significance to NIAMD:

An understanding of the effects of incorporating various types of lipids into phospholipids bilayers should contribute to the understanding of the properties of cell membranes, which contain a number of phospholipids. These studies may also conceivably contribute to the understanding of those membrane diseases which are characterized by the presence of abnormal lipids. The hysteresis behavior of the hexadecyl alcohol transitions may have a bearing on the mechanism of the "switching" processes occurring on stimulation of excitable cells.

Honors and Awards: None

Publications:

Gottlieb, M. H.: On the Rates of Exchange between Free and Bound Counterions in Polyelectrolyte Solutions. I. Electroosmotic Flows during Determinations made by an Electrical Transference Method. J. Phys. Chem., 75:1981-85 (1971).

Gottlieb, M. H.: On the Rates of Exchange between Free and Bound Counterions in Polyelectrolyte Solutions. II. An Explanation for the Anomalous Results obtained by the Electrical Transference Method. J. Phys. Chem., 75:1985-89 (1971).

Gottlieb, M. H.: On the Rates of Exchange between Free and Bound Counterions in Polyelectrolyte Solutions. III. A Demonstration That the Exchange Is Not as Slow as Indicated by the Electrical Transference Measurements. J. Phys. Chem., 75:1990-93 (1971).

Serial No. NIAMD-LPB-6
1. Physical Biology
2. Electrochemistry and
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The physical chemistry of membranes and complex membrane systems of biological interest

Previous Serial Number: NIAMD-LPB-7

Principal Investigator: Karl Sollner

Other Investigators: Gerald M. Shean

Cooperating Units: None

Man Years:

Total	2-2/3
Professional:	2
Other:	2/3

Project Description:

Objectives:

The physico-chemical study of membranes, and complex membrane systems and models with the ultimate aim of providing a rational basis for various complex membrane phenomena in living systems.

Methods Employed:

Conventional chemical-analytical and electrochemical methods, especially potentiometric measurements as reported previously.

Major Findings:

The central topic of investigation was the physical chemistry of liquid ion exchanger membranes. The studies on concentration cells with anion exchanger membranes were continued, and accurate and exhaustive data were assembled. Likewise, the previously reported work on bi-ionic potentials across anion exchanger membranes was continued, and expanded. In particular, systematic measurements were carried out under recently clearly defined conditions so that the accuracy and reproducibility of the experimental data is now ± 0.5% and better. This material has been prepared for publication. In addition, preliminary studies on poly-ionic potential across the same membranes have been carried out and show excellent agreement with the theoretical prediction.

The exploratory work on liquid cation exchanger membranes was further pursued. In particular, the search was continued for membranes which facilitate the construction of cells free of a variety of shortcomings; such cells are necessary for systematic physicochemical studies of a basic nature. The scarcity of literature on liquid cation exchangers and the difficulty of securing potentially useful strong acid compounds of adequate purity have made progress along this line rather time consuming. A variety of organic phosphates and sulfonates dissolved in several solvents were tested. Reasonably satisfactory concentration potentials were obtained with membranes consisting of dinonyl naphthalene sulfonic acid in o-nitrotoluene. However, due to micelle formation in the membrane phase the resistances of these membranes were much higher than desirable for the planned systematic investigation, and the water solubilities of the sulfonate salts are too high. Finally, it was discovered that dipicrylamine, 2,2', 4,4', 6,6' hexanitrodiphenylamine, a strong acid, when dissolved, e.g. in o-nitrobenzene, in its various salt forms, yields membranes which are suitable for systematic study. Membranes of various ion exchanger content in various solvents were systematically surveyed with respect to their electromotive behavior in concentration and bi-ionic cells. The currently available data show a striking parallelism to those reported last year for anion exchanger membranes.

Proposed Course of the Project

Systematic studies on concentration potentials with a variety of critical cations across dipicrylamine membranes; systematic studies on bi-ionic potentials across such membranes, also on poly-ionic potentials; continuation of the systematic work on anion exchanger membranes, particularly polyionic potentials. Continuation of the search for more desirable ion exchanger compounds.

Significance to NIAMD Research:

Movement of solvent and solutes is an important aspect of all life processes. This project tries to provide solidly founded, nonspeculative physicochemical background information on the functional behavior of membranes in general. At the same time, these membrane studies have furnished a new, now widely used tool for the electrometric determination of ion activities - the use of permselective membranes as membrane electrodes. It is perhaps also of interest that Sollner's "permselective" membranes are the prototypes of the ion exchange membranes which are used now in the electrodialytic desalination of sea and brackish water.

Publications: None

Serial No. NIAMD-LPB-7
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title:

1. Determination of the physical and chemical characteristics of complexes between retinal and phosphatidyl ethanolamines.

2. Construction of a model system to approximate the complex described and to determine a possible relationship to rhodopsin.

Previous Serial Number: NIAMD-LPB-8

Principal Investigator: Ralph G. Adams

Other Investigators: None

Cooperating Units: W. H. Jennings
N. E. Sharpless

Man Years:

Total:	1
Professional:	1
Others:	None

Project Description:

Objectives:

1. Complexes between phosphatidyl Ethanolamine (P.E. and retinaldehyde form spontaneously in vitro but only in anhydrous organic solvents. If these complexes are, as we believe, the chromophoric portion of the visual pigment rhodopsin, then they must exist in an aqueous surrounding. A thorough understanding of the structure and kinetics of the formation of the complexes is prerequisite to understanding how they exist in an aqueous environment. It is likely a hydrophobic layer surrounds the complex and its removal produces destruction of the latter causing "bleaching."

2. An obvious endpoint to seek is the construction of synthetic rhodopsin or functional analog. To accomplish that purpose physical, quantum mechanical and chemical models have been constructed and tested.

Major Findings:

1. Complex II, formed by a π-π interaction between an unsaturated bond of an EPG fatty acid and retinaldehyde, arises from the protonated Schiff's base of EPG and retinaldehyde (Complex I). Complex II has very complicated formation kinetics we have not been able to completely unravel. Recently, I have been able to isolate the Schiff's base, unprotonated and free of water; its transformation into Complex I, whose extinction coefficient is known, will enable us to determine the molecular absorption constant for the unprotonated Schiff's base. This latter is the key to forming Complex II. Since our present computer programs require only one more known quantity we should be able to unravel the kinetics by curve fitting technique.

We have clarified the discrepancy between the unprotonated Schiff's base classically absorbing at 365 nm and what we have shown is the unprotonated imine between EPG and Retinal which absorbs, both by calculation and empirically, at 340 nm. The presence of water of formation of the imine in some fashion increases the effective conjugation of the resultant molecule extending its absorption maximum into the red. Methanol will remove the conjugative effect, probably by hydrogen bonding the water. Some effect of the phosphate group is also present since the substitution of a sulfate moiety for it interferes with the effect of the methanol.

2. Incubation of Complex II with Bovine Serum Albumin results in slight increase in absorption at the 500 nm portion of the spectrum and the photosensitivity is apparently increased. The effect is minimal at this time but only one experiment has been tried.

Significance to NIAMD Research:

To elucidate the structure of rhodopsin, the visual pigment is an obviously important step in understanding vision.

Proposed Course of the Project:

1. Application of results of kinetic studies to formation of model compounds approximating rhodopsin.

2. Flash photolysis of model compounds of retinal and phospholipid.

3. Further experimentation with synthetic rhodopsin solutions.

4. Analysis of native rhodopsin in light of data now available concerning complexes.

Honors and Awards: None

Publications: None

Serial No. NIAMD-LPB-8
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Molecular structure determined by spectroscopic methods

Previous Serial Number: NIAMD-LPB-9

Principal Investigator: Edwin D. Becker

Other Investigators: Robert B. Bradley
William H. Jennings

Cooperating Units: H. Michael McIntyre (NIH Fellow)
Marjorie S. Malmberg (NIH Fellow)
Edwin Tucker (NIH Fellow)
T. C. Farrar (JEOL, Inc.)
D. M. Jerina (NIAMD-LC)
W. Friauf, T. Clem (DRS-BEIB)
C. Fisk (Georgetown University)
F. J. Brinley (Johns Hopkins University)
H. J. C. Yeh (NIAMD-LC)
E. Gross (NICHHD)

Man Years

Total:	4
Professional:	3
Other:	1

Project Description:

Objectives:

1. Development of an understanding of the forces within and between molecules, especially those of potential biological importance.

2. Development of spectroscopic methods for studying molecular structure and analyzing materials of chemical and biological interest.

Methods Employed:

The primary techniques used in this work are infrared spectroscopy (IR), Raman spectroscopy, and nuclear magnetic resonance spectroscopy (NMR). The three methods supplement each other in providing detailed information about the structure of molecules. In addition, the spectra are highly sensitive to the effects of molecular interactions. For studies of

hydrogen bonding high precision vapor pressure measures provide information complementary to that obtained by the spectroscopic techniques. Digital computer methods are used extensively.

Major Findings:

Our investigations of ^{13}C NMR spectra and the development of methods for such study advanced slowly, (1) with the completion of apparatus for 55 MHz pulse measurements using our superconducting magnet; (2) with the initiation of spectral studies of a cyclic peptide (nisin) and some specific lipids and phospholipids; and (3) with the completion of a series of tests to evaluate the advantages and limitations of multipulse methods such as DEFT. Much of the ^{13}C work has been published (see list of publications).

^{23}Na NMR has been used to study sodium in human erythrocytes and the results compared with results previously obtained for sodium in frog muscle. In blood cells the ^{23}Na signal is similar to that in non-cellular situations, which indicates that the high degree of intracellular organization of sodium postulated by some investigators for muscle and other cells is not necessary for active transport.

Hydrogen bonding of t-butanol in a hydrocarbon solvent is being investigated by IR, NMR and vapor pressure methods. The results are in accord with the presence of trimers and hexamers, but essentially no dimers.

Nuclear magnetic double resonance studies of ^{15}N-substituted oxaziridines have shown that the sign of the ring ^{13}C-^{15}N reduced coupling constant is negative, an unexpected result. Participation of the nitrogen lone pair of electrons in this and other couplings in these molecules is indicated.

The Building 2 time-sharing computer facility, covered for administrative purposes in this project, is becoming increasingly valuable to LPB and LMB, as more user programs are prepared and a larger number of scientists become familiar with the operation and capabilities of the computer system. IR and UV data can be taken routinely and some use has been made of the system in recording and analyzing CD and ORD data. A comprehensive status report of the computer system has been prepared to aid in planning future work.

Significance to NIAMD Research:

In this project we are attempting to obtain basic information on the structure and molecular interactions of nucleosides and their derivatives and to learn more about the nature of hydrogen bonding in these and other molecules. Such information may aid in an understanding of the relation between structure and function of nucleic acids and enzymes. In addition, the development of spectroscopic techniques applicable to molecules of biological activity and interest, such as steroids, alkaloids, pigments,

etc., aids in the structure elucidation of many natural and synthetic substances studied throughout NIH.

Proposed Course of Project:

We anticipate that major emphasis will continue to be placed on ^{13}C NMR studies, with the expected completion of the necessary decoupling and computer facilities.

The possibility of investigating by NMR the state of Rb^+ and Tl^+ ions in muscle and blood cells is being evaluated.

IR and NMR studies of hydrogen bonding in 2,4-dimethyl-3-ethyl-3-pentanol, a highly hindered alcohol will be initiated soon.

In spite of a concerted and largely successful effort to write up results of previously completed work, we still have manuscripts in preparation on (a) IR and Raman studies of 1-methyluracil, (b) NMR investigations of asymmetry in a series of cyanoacetates, (c) NMR determination of signs of coupling constants in nitromethane, and (d) the basis and application of Fourier transform spectroscopy. These papers should be completed soon.

Publications:

Shoup, R. R., Miles, H. T. and Becker, E. D.: Restricted rotation about the exocyclic carbon-nitrogen bond in cytosine derivatives. J. Phys. Chem. 76: 64-70, 1972.

Shoup, R. R., Becker, E. D. and McNeel, M. L.: An evaluation of the nuclear magnetic resonance total line shape analysis of uncoupled, exchanging two-site systems. J. Phys. Chem. 72: 71-78, 1972.

Farrar, T. C., Druck, S. J., Shoup, R. R. and Becker, E. D.: Temperature-dependent ^{13}C relaxation studies of small molecules. J. Am. Chem. Soc. 94: 699-703, 1972.

Axenrod, T., Pregosin, P. S., Wieder, M. J., Becker, E. D., Bradley, R. B. and Milne, G. W. A.: Nitrogen-15 nuclear magnetic resonance spectroscopy. Substituent effects on ^{15}N-H coupling constants and nitrogen chemical shifts in aniline derivatives. J. Am. Chem. Soc. 93: 6536-6541, 1971.

Shoup, R. R., Becker, E. D. and Farrar, T. C.: The driven equilibrium Fourier transform NMR technique: An experimental study. J. Magnetic Res. In press.

Becker, E. D., Shoup, R. R. and Farrar, T. C.: ^{13}C NMR spectroscopy: Relaxation times of ^{13}C and methods for sensitivity enhancement. Pure Appl. Chem. In press.

Becker, E. D.: Some recent developments in high resolution NMR. Appl. Spectr. In press.

Shoup, R. R. and Farrar, T. C.: ^{13}C magnetic relaxation rate studies of chloroform. J. Magnetic Res. In press.

REPORTED IN PRESS - 1971

Shoup, R. R. and VanderHart, D. L.: Effect of CH scalar coupling on ^{13}C transverse relaxation times. J. Am. Chem. Soc. 93: 2053-2054, 1971.

Becker, E. D., Bradley, R. B. and Axenrod, T.: Double resonance studies of isotope effects on nitrogen chemical shifts. J. Magnetic Res. 4: 136-141, 1971.

Becker, E. D.: A proposed scale for nitrogen chemical shifts. J. Magnetic Res. 4: 142-147, 1971.

Serial No. NIAMD-LPB-9
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Molecular structure, organization and intermolecular forces

Previous Serial Number: NIAMD-LPB-10

Principal Investigator: Elliot Charney

Other Investigators: Julie B. Milstien

Cooperating Units: Dr. J. M. Edwards, University of Connecticut
Dr. E. F. Rossomando, Brandeis University
Dr. Ulrich Weiss, NIAMD-LPB
Dr. Kiwamu Yamaoka, Hiroshima University, Japan
Dr. Herman Ziffer, NIAMD-LPB

Man Years:

Total:	3
Professional:	3
Other:	0

Project Description:

Objectives:

The general objectives of this research are to determine the role of molecular structure and of intermolecular forces in chemical, physical and biological phenomena by exploring the optical properties of molecules (spectroscopic absorption, optical rotatory dispersion, circular and linear electric-field induced dichroism).

Methods Employed:

The methods have all been described in previous reports including the methods which are unique or almost unique to this laboratory.

Major Findings:

1. Electric field induced dichroism of large molecules

   a. Polynucleotides (with Dr. Milstien): An electric dichroism study of polyribosadenylic acid (poly A) has been completed with the following results: (1) Double stranded poly A is flexible at

molecular weights (200,000-300,000 Daltons) at which DNA is rigid (reported earlier). (2) In the spectral region between 290 nm and 200 nm, there are four distinct transitions in poly A. The absorption band at 260 nm in fact consists of three separate electronic transitions, two in the plane of adenine bases near 270 and 250 nm and, one, the elusive but previously predicted out-of-plane $n \rightarrow \pi^*$ band near 230 nm. (3) The bases in poly A in solution are tilted from the perpendicular to the helix axis by at least the 10° found in poly A fibers by Rich, Davies et al. (4) The adenine bases retain their nearly perpendicular aspect with respect to the polymer backbone in both forms of double stranded poly A and in single stranded poly A as well, the latter confirming the conclusions drawn about single stranded poly A by Leng and Felsenfeld.

b. DNA (with Dr. Milstien and Dr. Yamaoka):

Electric Dichroism of Short Chain DNA: DNA chains at least as short as the hydrodynamic persistence length obtained by poly-D-lysine titration by Dr. Robert Clark, can be oriented in an electric field, and give values of dichroism approaching that, but lower than that expected for B-form DNA. Under conditions of very low salt (<.0025 M NaCl), the field strength dependence of the electric dichroism at low fields indicates the dominance of an orienting mechanism other than induced dipole moment, in contrast to results at higher salt concentrations (.005 M NaCl) or on samples of longer chain DNA at low salt.

c. DNA (with Dr. Yamaoka):

Preliminary examination of new data on carefully sonicated DNA, not only confirm our earlier results that the lower molecular fragments are rigid, but indicate that the out-of-plane component of the 260 nm transition moment is larger than expected from a first-order prediction under the assumption of a Watson-Crick-Wilkins structure.

d. Structure of bacteriophage fl (Dr. Milstien and Dr. Rossomando):

Previous work using electric birefringence and dichroism techniques have shown that the structure of the rod-shaped bacteriophage fl, which contains a circular single-stranded DNA wrapped in a coat of protein subunits, is mediated by a minor protein component located at one end of the particle. Further studies using the effect of temperature on the structure indicate that the intersubunit contacts are largely hydrophobic in nature. In addition, as the temperature is increased, there is an increase in the DNA base tilt occurring at temperatures well below that at which loss of viability occurs. This work has now been extended, using circular dichroism to monitor structural changes caused by increasing temperature, limited proteolytic digestion, and alkali treatment, to give results which

suggest that the DNA circle is packaged in protein, then twisted into a "coiled coil" to result in a rod-shaped structure.

2. Optical activity and electronic states of small molecules (with J. M. Edwards, U. Weiss and H. Ziffer)

   The chiral-optical effects of heteroannular cisoid dienes were previously attributed to the dissymmetry of the skewed diene chromophore as in the case of homoannular cisoid dienes and of transoid dienes. We have found that the sign of these effects for a small number of heteroannular cisoid dienes is opposite to that predicted from the "diene rule" (I). This inversion of sign is ascribed to the change in electronic properties of the diene when, as in these compounds, the dihedral angle about the central bond is very large compared to that of the homoannular dienes. This confirms recent theoretical treatments of the functional dependence of the rotatory strengths on the dihedral angle at large angles.

Significance to NIAMD Research:

Optical and electrical properties of small and large molecules helps elucidate their structures and the relationships between structure, reactivity, and the intermolecular forces.

Proposed Course of Project:

We will continue through single crystal spectroscopy and electric dichroism to attempt to discover the origin of the large angle which the electronic transition moment, supposedly in the plane of the constituent nucleic acids, appears to make with the perpendicular to the helix axis of DNA and poly A. In addition we plan to convert a microspectrophotometer to a dual instrument so that it can also be used as a microspectropolarimeter. With this instrument we will investigate a little explored phenomenon, the circular dichroism of oriented systems. In particular we expect to measure the CD of the pseudo-crystal of poly A recently produced by S. Zimmerman in the Laboratory of Molecular Biology. These measurements, in addition to providing a basic investigation of the phenomenon should enable us to help answer the question posed above and thus provide further insight into the physical and optical properties of the nucleic acids.

We also plan to use the electric dichroism technique to investigate a number of biologically interesting phenomena associated with protein behavior in transcription.

Publications:

Charney, E. and Tsai, L.: Spectroscopic examination of the lower excited states of α-diketones: Camphorquinone. J. Amer. Chem. Soc. 93: 7123-7132, 1971.

Milstien, J. B. and Rossomando, E. F.: Electrooptic studies of the effect of heat treatment on structure in bacteriophage f1. Virology 46: 655-662, 1971.

REPORTED IN PRESS - 1971

Charney, E.: Linear dichroism with special emphasis on electric field induced linear dichroism. In Cantoni, G. and Davies, D. (Eds.): Procedures in Nucleic Acid Research. New York, Harper and Row, 1971, pp. 176-204.

Charney, E. and Yamaoka, K.: Electric dichroism of DNA in solution. In Macromolecular Preprint (Vol. I). Boston, XXIII International Congress of Pure and Applied Chemistry, 1971, pp. 252-255.

Rossomando, E. F. and Milstien, J. B.: Electrooptic evidence for the control of the structure of bacteriophage f1 by a minor coat protein. J. Mol. Biol. 58: 187-195, 1971.

Serial No. NIAMD-LPB-10
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Conformation and electronic structure of biological molecules

Previous Serial Number: None

Principal Investigator: William A. Eaton

Other Investigators: None

Cooperating Units: Dr. Marvin Makinen, formerly NIDR-LB
Dr. Walter Lovenberg, NHLI-ET
Dr. Graham Palmer, University of Michigan

Man Years

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

The general aim of the research is to relate the stereochemistry and electronic structure of biological molecules, particularly proteins, to their biochemical reactivity and function.

Methods Employed:

In addition to conventional biochemical and spectroscopic methods, techniques have been developed for obtaining polarized spectra of microcrystals from the ultraviolet to near infrared wavelength regions.

Major Findings:

1. Heme proteins (with Dr. Makinen)

   a. Electronic structure of heme. Using microspectroscopic techniques the polarized single crystal absorption spectra of a large variety of hemoglobin and myoglobin derivatives has been investigated over a wide wavelength range (230-1200 mμ). The basic polarization of almost all of the well-known optical transitions of the common ferric and ferrous heme protein derivatives has now been established. The in-plane (x,y) polarization deriving from

porphyrin pi electron transitions dominates the spectra. However, several transitions are found to be polarized essentially perpendicular (z) to the porphyrin plane. Characterization of z-polarized transitions is particularly important, since there is a greater possibility of making definite spectroscopic assignments for testing and refining theoretical descriptions of heme electronic structure.

In addition to revealing "new" optical transitions, which are obscured in the isotropic (solution) spectra, the polarization ratio spectra demonstrate the kinds of deviations from perfect square symmetry of the heme chromophore that result from interaction with the axial ligands and surrounding protein.

b. Conformation. The investigation described above demonstrated that the dichroism of the Soret band is a reliable and extremely sensitive monitor of heme orientation in single crystals--heme rotations resulting from changes of axial ligands and/or oxidation state of 2-3 degrees being easily detectable. Since the heme is in close contact with the surrounding protein, changes in heme orientation must be accompanied by changes in protein conformation. The Soret dichroism results on horse hemoglobin indicate that the conformation of the heme environment can be correlated with the iron out-of-plane displacement. This result provides evidence for the Hoard-Perutz hypothesis that heme stereochemical changes "trigger" conformational changes in the protein that lead to cooperative oxygen binding. Changes in heme orientation in sperm whale myoglobin crystals are much less than in hemoglobin, suggesting that the protein environment of the heme in myoglobin is much less flexible than in hemoglobin.

2. Iron-sulfur proteins.

a. Ferredoxin and adrenodoxin (with Drs. Lovenberg and Palmer). A near infrared circular dichroism study of these electron transfer proteins has been completed. The identification of low-frequency, magnetic-dipole allowed d→d transitions establish that the reduced proteins contain a high spin ferrous ion in a distorted tetrahedral site. Furthermore, the frequencies of these optical transitions provides a critical test of, and confirms, the theory of the g = 1.94 electron paramagnetic resonance signal, which has been the major analytical tool for studying the biochemistry of these proteins in vitro and in vivo.

b. Rubredoxin (with Dr. Lovenberg). A polarized single crystal absorption study of oxidized rubredoxin has been completed. The crystal spectrum shows an axial distortion of the tetrahedral iron-cysteine complex which does not correspond to the axial distortion observed in the high resolution x-ray investigation.

Significance to NIAMD Research:

The elucidation of relationships between conformation and electronic structure of the active sites of proteins should lead to a much deeper understanding of how these molecules perform their biological function.

Proposed Course of Project:

We shall continue to investigate the electronic structure and conformation of heme proteins. From an examination of changes in heme orientation in other hemoglobin and myoglobin crystals we should be able to define what are the important structural features that lead to the different tertiary structural behavior of hemoglobin and myoglobin.

The polarization data from crystal spectroscopy, together with planned magnetic circular dichroism and low temperature measurements, should provide a comprehensive interpretation of the origin of heme protein spectra. Once theoretical calculations have been performed on the ground and excited states of oxyhemoglobin, this optical study should be able to distinguish between the various models that have been proposed for the geometry and electronic structure of the iron-oxygen bond.

Publications:

Eaton, W. A., Palmer, G., Fee, J. A., Kimura, T. and Lovenberg, W.: Tetrahedral iron in the active center of plant ferredoxins and beef adrenodoxin. Proc. Nat. Acad. Sci. 68: 3015-3020, 1971.

REPORTED IN PRESS - 1971

Lewis, T. P. and Eaton, W. A: Polarized single-crystal absorption spectrum of cytosine monohydrate. J. Am. Chem. Soc. 93: 2054-2056, 1971.

REPORTED IN PRESS - 1970

Eaton, W. A. and Charney, E.: Near infrared circular dichroism: d→d transitions in hemoproteins. In Chance, B., Lee, C. P. and Blasie, J. K. (Eds.): Probes of Structure and Function of Macromolecules and Membranes: Vol. 1, Probes and Membrane Function. New York, Academic Press, 1971, pp. 155-164.

Serial No. NIAMD-LPB-11
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Physical and Chemical Bases of Photoreception

Previous Serial Number: Same

Principal Investigator: W. A. Hagins

Other Investigators: Shuko Yoshikami
Hartmann Rüppel

Cooperating Units: Frances M. Hagins, Guest Worker, LPB-NIAMD
Kurt Heinrich, U. S. National Bureau of Standards

Man Years

Total:	3.25
Professional:	3.25
Other:	0

Project Description:

Objectives:

To outline the successive events by which light quanta absorbed in the receptors of animals lead to the transmission of information to the brain.

Methods Employed:

1. Measurements of currents, voltages, and impedances of the retina by conventional electronic methods.
2. Measurement of photochemical changes in rhodopsin in living cells by kinetic microspectrophotometry.
3. Analysis by digital computation of electrical signals produced by photoreceptors.
4. Investigation of the chemical structure of rhodopsin by spectrophotometry and amino acid analysis.
5. Electron microscopy of the cellular structure of photoreceptors.
6. Analysis of the ionic composition of the retina by electron probe microanalysis.

Major Findings:

1. Study has continued on the factors shaping the fast photoelectric charge displacement (FPV) caused when light is absorbed by rhodopsin in the plasma membranes covering the outer segments of vertebrate retinal rods. The decay of metarhodopsin I, an intermediate in the photolysis of rhodopsin, has been shown to be simultaneous with the production of the R_2 component of the FPV. The total charge displacement is equivalent to one electronic charge moving a distance equivalent to half the thickness of a typical lipid bilayer (35 Å) (Hagins and Rüppel).

2. A new light-induced ionic current, the "L" current, has been detected in the envelope membranes of rod outer segments of rats in solutions containing very low concentrations of Ca^{++} ions. This current is somewhat slower than the decay of metarhodopsin I but precedes the electrical events now believed to signal neural excitation in rods. The "L" current is suppressed by light adaptation of rods and by Ca^{++} applied externally to them. Thus one more piece of evidence now exists showing the similarity of the action of Ca^{++} and light on rods and implicating Ca^{++} as an internal excitatory transmitter in rods and cones (Yoshikami and W. A. Hagins).

3. Highly purified preparations of squid rhodopsin have been prepared by gel electrophoresis in sodium dodecylsulfate. The purified preparations, like those reported in 1971, possess an N-terminal glycine residue, consist of single peptide chains of molecular weights 50,000 ± 1,000 and contain some carbohydrate. Steps are being taken towards sequencing studies (F. Hagins).

Significance to NIAMD Research:

Progress in the understanding of molecular events underlying excitation of photoreceptors is obviously important in research on vision. In addition, it may provide a point of attack on the nature of permeability changes which occur in the surface membranes of electrically active cells, such as nerve and muscle, during transmission of nerve impulses and initiation of muscular contraction.

Proposed Course of Project:

Present experiments are directed towards answering the three questions:

1. What is the ionic mechanism of the currents produced by vertebrate photoreceptors?
2. What part do the kinetics of the rod responses to light play in shaping the overall function of the eye in dark and light adaptation?
3. What part do rhodopsin's photochemical reactions play in initiating the electrical responses of rods?

Publications:

Penn, R. D., and Hagins, W. A.: Kinetics of the photocurrent of retinal rods. Biophys. J., in press, June, 1972.

Hagins, W. A.: The Visual Process: Excitation of Primary Photoreceptor cells. (Review). Ann. Rev. Biophys. Bioeng. 1:in press, June, 1972.

Serial No. NIAMD-LPB-12
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Structure of paramagnetic molecules and their interaction with environment

Previous Serial Number: Same

Principal Investigator: Hideo Kon

Other Investigators: Ryo Hirasawa

Cooperating Units: Dr. Marvin W. Makinen, NIDR-LB
Dr. Julie B. Milstien, NIAMD-LPB

Man Years

Total:	1.3
Professional:	1.3
Other:	0

Project Description:

Objectives:

To investigate the electronic structure of transition metal ions and/or organic or inorganic free radicals incorporated in some biologically interesting systems and to obtain information concerning the behavior of the molecule and the state of the surroundings.

Methods Employed:

Electron paramagnetic resonance (epr) was the basic experimental means and molecular quantum theory the principal theoretical tool. The apparatus to produce the temperature below boiling nitrogen was frequently used. Other spectroscopic methods were employed when needed. Digital computers were used for spectral simulation and other numerical calculations.

Major Findings:

1. With Dr. Hirasawa (Continued from item 2 of 1970 report)

The polarized single crystal optical absorption of Cu(II)-doped histidine·HCl·H_2O combined with Gaussian analysis indicates that

there are three d-d transitions at 14 000, 12 800 and 11 100 cm^{-1}, which are mainly polarized along the c, b and a crystal axes, respectively. The result was interpreted consistently with the previously obtained electron paramagnetic resonance (EPR) data to give the one-electron orbital sequence, $d_{z^2} > d_{x^2-y^2} > d_{yz} > d_{xy} > d_{xz}$, with the unpaired electron in the d_{z^2} orbital. Also on this basis approximately D_2 symmetry was obtained for the interstitial Cu(II) site.

2. Epr absorption of molecular oxygen in the ground triplet state in condensed phases was observed for the first time. So far the molecule had been studied in a gas phase at pressures less than ∿ 10 mmHg, because of the strong pressure broadening. The well-defined absorption observed this time at the x-band microwave frequency comes from oxygen molecules isolated in the matrix of N_2, CO, etc., at or below ∿ 10° K, and was attributed to the orientation with the molecular axis approximately perpendicular to the external magnetic field of ∿ 12 000 gauss. The absorption width showed a marked matrix and concentration dependency. The isotope effect due to $^{18}O_2$, and the nuclear hyperfine structure due to ^{17}O (enriched) were also detected.

3. With Dr. Makinen and Dr. Milstien (Continued from item 2, 1969-1970 report)

   Comparative study of the EPR spectra of the nitric oxide derivatives of human, horse and bovine hemoglobin (Hb) and the respective complex with human haptoglobin (phenotype 2-1, Hp_{2-1}) showed that guaiacol, the substrate for peroxidatic assay, denatures Hb and that Hp_{2-1} binding renatures it to roughly the same degree regardless of the species origin of Hb used. The result is in agreement with the trend in the measured peroxidatic activity of Hb-Hp_{2-1} complexes in that no significant species dependence was observed. This indicates that the changes of electronic structure in heme iron caused by Hp binding do not vary with species origin of Hb. Such species-dependent variation, however, does exist in other areas of the Hb structure as evidenced by ORD and CD measurements and by the transient electro-optic studies.

Significance to NIAMD Research:

These investigations contribute basic information toward understanding interactions involving metal ions, small organic molecules and the protein in more complicated and biologically significant systems.

Proposed Course of Project:

Investigations of the relationship between the function and the structure of metalloproteins will be continued, along with the study of the structure of small paramagnetic molecules.

Publications:

Hirasawa, R. and Kon, H.: Electron paramagnetic resonance and polarized absorption spectra of Cu(II) doped single crystal of L-histidine hydrochloride monohydrate. J. Chem. Phys. In press.

REPORTED IN PRESS - 1971

Miller, J. H., White, F. H., Jr., Riesz, P. and Kon, H.: Distributions of free radicals among amino acids from lyophilized ultraviolet-irradiated proteins. Photochem. Photobiol. 14: 577-588, 1971.

Serial No. NIAMD-LPB-13
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Molecular Dynamics

Previous Serial Number: Same

Principal Investigator: Ira W. Levin

Other Investigators: None

Cooperating Units: William C. Harris, NIAMD-LPB and Furman University, Greenville, South Carolina
O. W. Adams, National Science Foundation
S. Abramowitz, National Bureau of Standards
A. Müller, University of Dortmund, Germany

Man Years:

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

An understanding of the vibrational characteristics of polyatomic systems through the analysis of Raman and infrared spectra, the determination of reliable intramolecular force fields and the interpretation of molecular charge distribution (intensity) parameters.

Methods Employed:

Various laser Raman and infrared spectroscopic procedures provided the required data for the vibrational analyses. Both the intramolecular force field determinations and large molecular orbital calculations were necessarily performed on the digital computer.

Major Findings:

1. A liquid helium cryostat was specifically designed for obtaining laser-Raman matrix spectra in the temperature range 4-20° K. Raman spectra of matrix isolated CCl_4 were readily obtained from samples with dilutions in argon of 1:400 to 1:600 mole ratios. The spectrum of the ν_4 mode of matrix isolated CCl_4 exhibits a splitting pattern

which is interpreted in terms of the chlorine isotope effect. Since small isotopic frequency shift information provides an effective constraint on the intramolecular force field, the matrix data are employed in assigning limits to the crucial stretch-bend interaction force constant. (Levin and Harris)

2. As a consequence of the dynamic Jahn-Teller effect, ReF_6 exhibits unusually low vibrational frequencies for the $\nu_2(e_g)$ and $\nu_5(f_{2g})$ fundamentals in comparison with the analogous modes of the normal 5d hexafluoride molecules. As an alternative to electronic spectroscopic techniques, the utility of model force fields toward estimating the unperturbed Jahn-Teller modes was examined. (Levin, Abramowitz and Müller)

3. In a continuing program for exploring low temperature Raman techniques, the spectra of polycrystalline acetone-d_0 and acetone-d_6 were investigated. A number of the methyl vibrations that are obscurred in the spectra of gaseous and liquid phase samples are assigned on the basis of the solid films. The interesting low frequency methyl torsional vibrations, not usually found in Raman spectra, were assigned from combination bands observed in the "mid-frequency" spectral region. (Levin and Harris)

4. Specific methods for simplifying the vibrational secular determinant were critically examined in an effort to understand more fully the physical basis of several commonly used approximations. (Müller, Mohan, Schmidt and Levin)

5. As part of our long range program for interpreting the vibrational dynamics of strained bicyclic ring systems, detailed infrared and Raman spectra of norbornane and norbornadiene were obtained for the gaseous, liquid and solid states. Descriptions of the normal modes and their coupling properties were based upon valence force field calculations. (Levin and Harris)

Significance to NIAMD Research:

Since experimentally determined vibrational frequencies and charge distribution parameters reflect the sum of several subtle electronic and nuclear contributions, force field and molecular orbital calculations permit both quantitative and qualitative descriptions of the dominant terms and create a link between empirical information and bond properties.

Proposed Course of Project:

In recent years, various investigators have demonstrated the practicality of calculating minimum energy conformations and strain energies from hydrocarbons and related systems. The success, however, of accurately determining structures by this method requires a moderately

detailed knowledge of representative potential functions. For this reason, the general method is presently limited to systems that are not highly strained. We intend to investigate, from a spectroscopist's viewpoint, the importance of the potential function in energy minimization calculations and to apply our present understanding of bicyclic molecules to more general calculations of strained systems.

Publications:

Harris, W. C., and Levin, I. W.: Raman spectra of polycrystalline isobutene-d_0, isobutene-d_6 and isobutene-d_8. J. Mol. Spectrosc. 39: 441-453, 1971.

Levin, I. W., and Adams, O. W.: Measurement and calculation of the absolute infrared intensities of PF_3. J. Mol. Spectrosc. 39: 380-391, 1971.

Levin, I. W., and Harris, W. C.: Intramolecular exchange in SF_4. J. Chem. Phys. 55: 3048-3049, 1971.

Levin, I. W.: Calculation of the infrared spectrum of SF_4 using CNDO/2 techniques. J. Chem. Phys. 55: 5393-5400, 1971.

Levin, I. W., Abramowitz, S., and Müller, A.: Jahn-Teller Vibrations of ReF_6. J. Mol. Spectrosc. 41: 415-419, 1972.

Harris, W. C., and Levin, I. W.: Raman spectra of polycrystalline acetone and acetone-d_6. J. Mol. Spectrosc. In press.

Levin, I. W.: Laser Raman Spectroscopy. In Johnston, J. W., Jr., Moulton, D. G. and Turk, A. (Eds): Advances in Chemoreception. New York, Appleton, Century, Crofts. In press.

REPORTED IN PRESS - 1971

Harris, W. C., and Levin, I. W.: Direct observation of the torsional vibrations in isobutylene, isobutylene-d_6 and isobutylene-d_8 by Raman scattering. J. Chem. Phys. 54: 3227-3229, 1971.

Levin, I. W., and Abramowitz, S.: Isotopic splitting in matrix isolated BF_3. Chem. Phys. Letters 9: 247-248, 1971.

Serial No. NIAMD-LPB-14
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Electronic and molecular structural investigations

Previous Serial Number: Same

Principal Investigator: Ruth McDiarmid

Other Investigators: None

Cooperating Units: None

Man Years

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

To describe the electronic and molecular structures of ground and excited states of simple molecules.

Methods Employed:

The measurement and analysis of electronic absorption spectra, predominantly in the ultraviolet. The calculation, with digital computers, of energy levels and geometries to correlate with and further explain the experimental observation.

Major Findings:

1. The spectrum of liquid ethylene was examined. The previously reported origin of the V←N system was shown to be an artifact arising from the presence of O_2 in the sample. The newly located origin is in agreement with that estimated for the molecule in the vapor phase.

2. The absorption system of diethyl ether around 1900 Å was investigated. At dry ice temperature the diffuse bands split into many sharp vibronic bands. Analysis of these and of those obtained for the perdeuterated compound has lead to an assignment of most of the observed bands. If a more detailed spectrum can be obtained, a molecular orbital assignment of this transition may be possible.

3. Further analysis of the ethylene spectrum suggests that the extra bands of deuteroethylene are sequence bands (bands arising from vibrationally excited levels of the ground electronic state).

Significance to NIAMD Research:

This research project seeks to understand the electronic changes a molecule undergoes on absorbing light and, more significantly, the structural alterations that a given electronic change can cause. This study can, if fruitful, provide a basis for a physical interpretation of the relative spectroscopic results obtained in other, more general studies.

Proposed Course of Project:

1. The spectra of liquified methyl- (and perhaps fluoro-) substituted ethylenes will be examined to investigate the effect of substitution on the origin of the V←N ($\pi^*\leftarrow\pi$) absorption system. If possible, this investigation will include a study of the T←N (singlet-triplet) absorption system of these molecules.

2. The ultraviolet spectra of WF_6 and MoF_6 will be studied to (1) locate their Rydberg transitions and (2) help in the analysis of ReF_6.

3. If suitable instrumentation is available, the $\sim$ 1890 Å transition of diethyl ether will be restudied in greater detail.

4. We will attempt to obtain a low temperature matrix spectrum of ethylene to help in the analysis of the vibronic structure of the V←N ($\pi^*\leftarrow\pi$) transition.

Publications:

McDiarmid, R.: On the 1795 Å transition of 2-methylpropene (isobutene). J. Chem. Phys. 55: 2426-2433, 1971.

McDiarmid, R.: Origin of the V←N transition of liquid ethylene. J. Chem. Phys. 55: 4669-4670, 1971.

REPORTED IN PRESS - 1971

McDiarmid, R.: Jahn-Teller effects in the $^2E_{(5/2)g} \leftarrow {}^2G_{(3/2)g}$ transition of rhenium hexafluoride. J. Mol. Spectrosc. 38: 495-502, 1971.

McDiarmid, R.: Higher electronic states of ReF_6. J. Mol. Spectrosc. 39: 332-339, 1971.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Mechanisms of energy transfer at cellular sites of photochemical action.

Previous Serial Number: NIAMD-LPB-15

Principal Investigator: Rodney A. Olson

Other Investigators: None

Cooperating Units: None

Man Years

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

To interpret the mechanisms of energy transfer and allied metabolic steps at cytological sites of photochemical action. To determine the role of chromophore orientation in laminar photoreceptors (chloroplasts, etc.) and in crystalline pigment-protein systems and paracrystalline pigment-lipid systems simulating the organized structure of the photosynthetic apparatus.

Methods Employed:

Low temperature (77°K) microspectrometry is used to sharpen spectra and reveal components which are obscured by vibrational band-broadening at room temperature. The microcryostat-spectrometer system described in the previous report has been updated in design and construction in terms of motion and adjustment stability. Improvements in the optical system include the design and use of impulse solenoid shutters which at 20 hz. control the sequence of events for sample, hold, and spectral data acquisition and transformation. A Wollaston prism now separates the polarization images and when appropriately oriented it compensates for the inherent polarization caused by the monochromator grating.

The electronic on-line digital timing and data transforming circuit is still in the building and testing stage in the hands of Biomedical Engineering at NIH. Current observations and measurements are therefore manual.

Major Findings:

There are some features inherent to cryogenic microspectrophotometry which limit the degree to which the high quality of similar measurements at room temperature can be approached. Immersion objectives are prohibited and resolution is limited by the resulting decrease to the numerical aperature practical for dry optics (from 1.40 down to 0.65). Further image degradation is caused by the thick windows of the vacuum shroud and by the formation of ice crystals in and around the specimen. The effect of the former can be minimized by corrective elements. Ice crystal formation can be minimized by adding dimethyl sulfoxide (10%) to the aqueous mounting medium. This reagent encourages the formation of vitreous ice. Under these conditions image quality is sufficient for current observations of Euglena and Mesotaenum chloroplasts but for more quantitative measurements a much larger organelle would be desirable. Much larger chloroplasts exist in other species and one with appropriate geometry (stacked parallel lamellae) is sought here. Observations and measurements of polarized spectra now indicate that the oriented pigment is a form of chlorophyll A absorbing maximally near ~685 nm. At room temperature the polarized absorption appears in the far red spectral region (695 nm) since only the long wavelength "toe" of the absorption of this form is not "optically diluted" by random oriented form of chlorophyll A absorbing at lower wavelengths. Low temperature uncovers more than this "toe" and indicates that the 685 absorbing chlorophyll A is an antenna pigment which through domains of parallel orientation and interaction funnels excitation energy to reaction centers or trapping sites involved in photochemical oxidation of water (chl 685 is currently considered a participant in the oxygen evolving process of photosynthesis).

Proposed Course of the Project:

Continuation of the cryogenic polarization spectral measurements described above in the direction of resolving by fluorescence excitation spectra, energy trapping sites and the effects of bound electron donors and acceptors. An attempt to find a large and flat chloroplast more appropriate for such measurements will be initiated among marine flora during fiscal 1973.

Publications: None

Serial No. NIAMD-LPB-16
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Spectral, physical-chemical and photochemical properties of biologically active substances

Previous Serial Number: Same

Principal Investigator: Norman E. Sharpless

Other Investigators: None

Cooperating Units: Frederick S. Brackett, Consultant, LPB-NIAMD
Ralph G. Adams (LPB-NIAMD)
William H. Jennings (LPB-NIAMD)
Robert B. Bradley (LPB-NIAMD)
James A. Ferretti (PSL-DCRT)

Man Years

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives: To investigate such molecular factors as structure, quantum parameters or physical properties in relation to the biological or pharmacological activities of the molecules.

Methods Employed:

These investigations utilize various forms of spectrophotometry (visible, UV, IR, NMR, EPR and fluorescence) which distinguish energy levels, and ORD, CD, gas chromatography and dielectric properties which distinguish other molecular properties, as well as theoretical quantum chemical calculations. Extensive use of computers is required to reach these objectives.

Major Findings:

Photochemistry of the Vitamin D System (with Dr. Brackett)

Computer analysis of steroid data has shown significant differences in

the photochemical behavior of steroids related to Vitamin D at the long wavelengths common to solar radiation from that usually studied at shorter wavelengths of the ultraviolet.

Retinaldehyde-Phospholipid Interactions (with Dr. Adams and Mr. Jennings)

A quantum mechanical investigation of retinaldehyde and a series of related nor-methyl compounds indicates that the three methyl groups of retinaldehyde are the requisite number to confer the proper level of instability upon retinaldehyde. The data indicate that an intra-molecular pi-pi complex in retinaldehyde-phosphotidyl ethanolamine Schiff base may in truth be the visual pigment.

Spectral studies of this complex and related compounds under anhydrous and ordinary conditions show differences which can be interpreted to mean that this class of compounds (Schiff bases) as ordinarily prepared are in fact hydrates, hydrogen bonded to a molecule of water. A specific effect of an intrinsic phosphate group on such molecules seems to be required to free the molecule from the water. The consequences of such a property in relation to the visual process is under consideration.

Nulcear Magnetic Resonance of Heterocyclic Fused Ring Compounds (with Mr. Bradley and Dr. Ferretti)

The computer analyses of these compounds has been continued. In general, chemical shifts are functions of electron distributions and degree of planarity of the molecules.

Therapeutic Interference

Experiments to introduce a spin label into DNA are continuing. If the DNA is in the double helix form, the spin label is unable to penetrate to the base to interact.

An important tool in binding studies is fluorescence spectrophotometry, if the probe be fluorescent, which trypaflavin is. However, this may lead to complications since the nitroxide radical was found to quench the fluorescence of trypaflavin. Quantitative studies of this effect are underway using the thiocyanate of a nitroxide. Isolation of the molecular fragments which may cause quenching (thus far, the CNS group) show that the thiocyanate ion (CNS^-) is a quite good quencher in aqueous solution; k_Q° ($\mu = 0$) = 81.5 for [TR] = 1.46 X 10^{-6} M and the slope of the ln k- μ curve is -1.0 as required by the Debye-Hüchel theory for a 1:1 electrolyte of opposite charges in collisional quenching. On the other hand, the neutral CNS (n-Butyl thiocyanate) does not quench the fluorescence of trypaflavine up to a concentration of 0.10 molar.

Significance to NIAMD Research:

A proper understanding of the behavior of biologically active substances at the molecular level is absolutely necessary for the understanding and interpreting of their action at the cellular and organ level in order to evaluate their contributions to both healthy and diseased states.

Proposed Course of Project:

Possible retinaldehyde-protein interactions will be studied, using model tripeptides containing both lysine and tryptophane, for information regarding other possible sources of the 400 nm absorption peak in the visual pigment.

The binding of therapeutic agents to spin-labeled DNA will be continued.

Publications:

REPORTED IN PRESS - 1971

Ferris, J. P., Boyce, C. B., Briner, R. C., Weiss, U., Qureshi, I. H., and Sharpless, N. W.: Lythraceae alkaloids X. Assignment of absolute stereochemistries on the basis of chiraloptical effects. J. Am. Chem. Soc. 93: 2963, 1971.

Serial No. NIAMD-LPB-17
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Chemistry and biosynthesis of natural compounds, and instrumental methods used in their study.

Previous Serial Number: Same

Principal Investigator: Ulrich Weiss

Other Investigators: Fatima N. Johnson

Cooperating Units: Prof. C. A. Salemink, University, Utrecht, The Netherlands
Dr. R. J. J. Ch. Lousberg, University, Utrecht, The Netherlands
Dr. Keith S. Brown, Jr., Centro de Pesquisas de Produtos Naturais, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brasil
Dr. J. M. Edwards, School of Pharmacy, University of Connecticut, Storrs
Dr. S. Liaaen Jensen, Department of Chemistry, Norwegian Polytechnic Institute, Trondheim, Norway
Dr. G. W. A. Milne, NHLI-LC
Dr. J. V. Silverton, NHLI-LC

Man Years

Total:	1.3
Professional:	1.3
Other:	0

Project Description:

Objectives:

Isolation in pure form, and elucidation of the chemical constitution of a variety of naturally occurring substances of chemical, biosynthetic, or biological interest. Study of instrumental techniques used in such work. Corroboration of chemical structure by total synthesis.

Methods Employed:

Standard procedures of organic chemistry, and instrumental methods (UV, IR, NMR spectroscopy). For measurement of optical rotatory dispersion and circular dichroism, the Cary 60 spectropolarimeter and its CD attachment are used.

Major Findings:

In cooperation with Dr. J. M. Edwards, the elucidation of the chemical structure of two pigments from the flowers of Lachnanthes tinctoria (Haemodoraceae) has been completed. These pigments are naphthoquinone methides related to the 9-phenylphenalenone (9-phenylperinaphthenone) pigments found in other parts of the plant (roots, pericarp), but differ from them by replacement of carbon atom 5 of the phenalenone ring system by oxygen or nitrogen, respectively. They are naphthoquinone methodes.

Tracer work on the biosynthesis of the phenalenones in Lachnanthes is being continued, and evidence for photodynamic action of some of the constituents of the plant has been obtained. Such activity had been suspected on the basis of an observation recorded in Darwin's "Origin of Species," but had never been proven.

In cooperation with Dr. K. S. Brown, the chemical structure of the two red-fluorescing trace constituents of Aphis nerii mentioned in last year's report has been established. Unexpectedly, they have turned out to be naphthoquinone methides. The fluorescing compounds are formed very readily by reaction of the aglycones of the two major glucosidic constituents of the insect with biacetyl (work by Drs. K. S. Brown and P. M. Baker, Rio de Janeiro). Both these aglycones are derived from 1,3,8-trihydroxynaphthalene. Since quinone methides in general show antitumor activity, this synthesis, and our observation of the occurrence of such compounds in a plant and in an insect, may be of interest for cancer chemotherapy; samples have been submitted for screening tests.

Work on the peculiar color dimorphism shown by the aphid, Macrosiphum liriodendri, has been completed in cooperation with Dr. S. Liaaen Jensen. The green aphids have been found to contain, besides the ubiquitous β-carotene, two novel carotenes in which one or both β-ionone rings of β-carotene are replaced by the γ-ionone system. The pigment with two such rings was new; its structure has been proved in Dr. Jensen's laboratory by total synthesis.

The pink form contains, besides β-carotene, the previously known γ-carotene (one β-ionone ring and one non-cyclic end-group of the type occurring in the well-known tomato pigment lycopene), and the red carotenoid torulene, which is a mono-dehydro-γ-carotene previously found in a red yeast; two other red pigments from the aphid were too unstable for complete elucidation of their structure; one of them seems to be identical with mono-dehydrolycopene.

The green aphids thus characteristically synthesize pigments with the γ-ionone ring, the pink ones dehydro derivatives of carotenoids with the non-cyclic lycopene end group.

The observation that both green and pink forms contain pigments previously found only in microorganisms suggests that the color dimorphism of this

aphid species (and of a number of related ones), which has long puzzled biologists and geneticists, may be due to differences in the nature or biosynthetic activities of the microsymbionts generally present in aphids, rather than to differences in the insects themselves.

The colorless metabolite isolated by Dr. Weisgraber from the deep blue mycelium of the basidiomycete (higher fungus) Corticium caeruleum has been shown by x-ray crystallography (Drs. Milne and Silverton) to be identical with the previously known leucohexamethyl ether of thelephoric acid, a well-known constituent of certain higher fungi and lichens. The leuco ether had not been observed before as a naturally occurring compound.

The structure of the previously known pigment cercosporin (from the mold Cercosporina kikuchii, a pathogen of soy bean) has been elucidated (R. Lousberg). Cercosporin is a dihydroxy-perylenequinone, like the pigments from the mold Elsinoe which have been studied in our laboratory, but it is unique in having a methylene-dioxy group, $-O-CH_2-O-$, attached to carbons 1 and 12 of the perylene system in such a way that a seven-membered ring results. The methylene dioxy group is common in natural compounds but very rare in quinones, and in other natural compounds invariably forms part of a five- or six-membered ring.

Several thiolcarbamates of the general formula $Ar-NH-CO-CH_2-S-CONH_2$, prepared by me years ago, had been found during World War II to show significant antimalarial activity in some of the tests then in use. With the renewed interest in antimalarials caused by the emergence of drug-resistant strains of Plasmodia, it seemed worthwhile to re-examine the possible value of these compounds. Dr. F. N. Johnson prepared two previously known and two new substances of this type, which have been submitted to the Walter Reed Army Medical Center for screening tests.

Proposed Course of Project:

Research on the chiroptical effects of non-planar conjugated dienes is to be resumed, particularly with a view towards explaining the anomalies observed in very strongly skewed heteroannular cisoid dienes (see report for 1971). Work towards the total synthesis of elsinochrome A is to be continued, as is the study of the constituents of Lachnanthes and their unusual biosynthesis and interesting photosensitizing action. Work on naphthoquinone methides should be pursued, if the results of screening tests for antitumor activity (now under way) should warrant this.

Publications:

Weisgraber, K. H. and Weiss, U.: The mode of formation, and some reactions of 2,3,6,7-tetramethoxy-9,10-dimethylphenanthrene. Can. J. Chem 49: 2366-2369, 1971.

Brown, K. S., Jr. and Weiss, U.: Chemical constituents of the bright orange aphid, Aphis nerii Fonscolombe. III. Two naphthoquinone methides

of unusual structure and chemical behavior. Tetrahedron Letters: 3501-3504, 1971.

Weisgraber, K. H., Lousberg, R. J. J. Ch. and Weiss, U.: The chemical basis of the color dimorphism of an aphid, Macrosiphum liriodendri Monell, and a locust, Amblycorypha sp.; novel carotenoids. Experientia 27: 1017-1018, 1971.

Lousberg, R. J. J. Ch., Weiss, U., Salemink, C. A., Arnone, A., Merlini, L. and Nasini, G.: The structure of cercosporin, a naturally occurring quinone. Chem. Commun.: 1463-1464, 1971.

Weiss, U., Whalley, W. B. and Karle, I. L.: The conformation of levopimaric acid and related dienes. Chem. Commun.: 16-17, 1972.

Weisgraber, K. H. and Weiss, U.: Pigments of Elsinoe species. Part VI. A simple synthesis of a related perylene quinone. J. Chem. Soc. (C): 83-87, 1972.

Edwards, J. M. and Weiss, U.: Quinone methides derived from 5-oxa- and 5-aza-9-phenyl-1-phenalenone in the flowers of Lachnanthes tinctoria (Haemodoraceae). Tetrahedron Letters. In press.

Edwards, J. M., Schmitt, R. C. and Weiss, U.: Biosynthesis of a 9-phenylperinaphthenone by Lachnanthes tinctoria (Haemodoraceae). Phytochemistry. In press.

Charney, E., Edwards, J. M., Weiss, U. and Ziffer, H.: Inverse sign of the chiral-optical effects of non-planar heteroannular cisoid dienes. Tetrahedron. In press.

Andrewes, A. G., Kjesen, N., Liaaen Jensen, S., Weisgraber, K. H., Lousberg, R. J. J. Ch. and Weiss, U.: Animal carotenoids. 6. Carotenes of two colour variants of the aphid, Macrosiphum liriodendri; identification of natural γ,γ-carotene. Acta Chem. Scand. 25: 3878-3880, 1971.

REPORTED IN PRESS - 1971

Brown, K. S., Jr. and Weiss, U.: Chemical constituents of the bright orange aphid, Aphis nerii Fonscolombe. II. Structures of some minor constituents. Anais Acad. Brasil. Ciências 42 (Supplemento): 205-210. 1970.

Ferris, J. P., Noyce, C. B., Briner, R. C., Weiss, U., Qureshi, I. H. and Sharpless, N. E.: Lythraceae alkaloids. X. Assignment of absolute stereochemistries on the basis of chiraloptical effects. J. Amer. Chem. Soc. 93: 2963-2968, 1971.

Weiss, U., Edwards, J. M. and Ziffer, H.: Some photochemical reactions of 9-bromophenanthrene. Austral. J. Chem 24: 657-660, 1971.

Serial No. NIAMD-LPB-18
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Titles:

1. A study of photoreactions and the mechanisms by which these reactions occur.
2. A study of the interdependence of CD (or ORD) curves and the structure and stereochemistry of dienes.
3. A study of the induced CD bands in optically inactive compounds by optically active solvents.

Previous Serial Number: Same

Principal Investigator: Herman Ziffer

Other Investigators: Jeffrey I. Seeman

Cooperating Units: Donald M. Jerina (NIAMD-LC)
D. T. Gibson, University of Texas

Man Years:

Total:	2
Professional:	2
Other:	0

Project Description:

Objectives:

1. To determine the absolute stereochemistry of (+) cis 2,3-dihydroxy-1-methylcyclohexa-4,6-diene, a microbial metabolite of toluene. To compare similar metabolites of the same enzyme system in an effort to learn more about the enzyme-substrate complex.
2. To examine the photochemistry of a number of β,γ-unsaturated ketones in order to learn more about the mechanism and stereochemistry of their reactions.
3. A study of the CD bands of substituted benzophenones induced by optically active solvents.
4. An examination of the optical activity of several cyclic cisoid dienes (in eight membered rings) in order to determine the effect of large angles on the sign of the long wavelength CD band.

Methods Employed:

The Cary 60 spectropolarimeter has been used to obtain ORD and CD measurements. Standard organic chemical techniques were employed for the synthesis and proof of structure of a number of compounds. Along with the usual spectroscopic measurements (NMR, IR and UV) we have used the Varian time averaging computer in conjunction with our NMR equipment to obtain several important NMR measurements necessary for structure determinations.

Major Findings:

1. Chemical and spectroscopic analysis of several dihydrodiols obtained from a Pseudomoras putida mutant grown on aromatic substrates (toluene ethylbenzene, and 4-fluoro toluene) indicated that the relative and absolute stereochemistry of those metabolites about the hydroxyl groups were the same.
2. The CD spectra of the above mentioned dihydrodiols were found to exhibit a strong band at $\sim$ 210 mμ opposite in sign to the longer wavelength band. The sign of the band had been predicted by theory but it had previously not been observed.
3. The peak of the CD curve of 1-ethyl-2,3-dihydroxy-$\Delta^{4,6}$-cyclohexadiene did not correspond to the absorption maximum (λ_{max}). This discrepancy has been rationalized by postulating the presence of a conformational equilibrium in which λ_{max} differs between the two conformers. A study of effect of solvent on the CD spectra has shown that both λ_{max} and the CD spectra change with solvent. It has been possible to fit the CD spectra using two bands of opposite sign separated by $\sim$ 5 nm. These results suggest that in an appropriately substituted diene the structure of the photoproduct could be controlled by monochromatic irradiation in the absorption band.
4. In examining the photochemistry of 5α-vinyl-A-nor-cholestan-3-one we have shown that irradiation under singlet conditions leads to photoepimerization. The only previous example of a β,γ-unsaturated ketone that photoepimerized proceeded via a triplet intermediate.

Significance to NIAMD Research:

Information on the absolute stereochemistry of metabolites will assist in developing models which describe the way substrates attach to enzymes and the course of the reaction.

The increased knowledge derived from studies of the mode and mechanism of photochemical reactions permit the synthesis of compounds that are difficult to prepare by other means.

Proposed Course of Project:

1. To determine the absolute stereochemistry of (+)-cis-2,3-dihydroxy-1-methylcyclohexa-4,6-diene.
2. To determine the relative stereochemistry of the trans diols, 1-methyl-2,3-dihydroxycyclohexane.
3. To continue the studies of the photochemical reactivity (singlet and triplet) of β,γ-unsaturated ketones.

Publications:

Charney, E., Edwards, J. M., Weiss, U. and Ziffer, H.: Inverse sign of the chiral optical effects of non-planar heteroannular cisoid dienes. Tetrahedron 28:973, 1972.

REPORTED IN PRESS - 1971

Weiss, U., Edwards, J. M. and Ziffer, H.: Some photochemical reactions of 9-bromophenanthrene. Austral. J. Chem. 24: 657-660, 1971.

Serial No. NIAMD-LPB-19
1. Physical Biology
2. Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Effects of altitude (hypoxia), exercise, and other environmental stresses on physiological, biochemical and pathological mechanisms in animals.

Previous Serial Number: Same

Principal Investigator: Paul D. Altland

Other Investigators: Mr. H. F. Brubach, Mr. M. G. Parker, Dr. R. G. Sellner, and Dr. M. P. Dieter

Cooperating Units: Dr. Benjamin Highman, Section on Pathologic Anatomy, Laboratory of Experimental Pathology, NIAMD, LEP-2 A.

Man Years (July 1971 through June 1972)

Total:	5.5
Professional:	3.5
Other:	2

Project Description:

Objectives:

To determine physiological, biochemical, and pathological changes during exposure to hypoxia induced by decompression or by low oxygen concentrations in nitrogen. To determine the effects of different degrees of hypoxia and polycythemia and associated graded tissue changes, particularly myocardial lesions, on exercise performance at sea level. To determine the mechanism of action of cross adaptation between altitude acclimation and exercise and between cold-acclimation and hypoxia administered at room temperature (Dr. Altland). To determine the mechanism involved in the production of deleterious effects of decompression hypoxia upon gestation (Dr. Sellner).

Methods Employed:

Biochemical analysis of serum enzymes and metabolic end products. Observations of respiration, body temperature and exercise performance. Histopathological methods for demonstration of fatty and necrotic changes in vital organs. Two altitude chambers, an animal exerciser, a gas chamber and a cold room.

Major Findings:

Exposure of rats to 18,000 ft. (Baro. Press. 379 mm Hg; pO_2 79 mm Hg) 5 hours daily for 6 weeks induced severe polycythemia (Hematocrit 76) but had no adverse effect on exercise performance at sea level. Severe polycythemia (Hematocrit 69) induced by oral administration of cobalt for 11 weeks likewise did not alter exercise performance. Except for polycythemia, no other tissue abnormalities were observed. In rats exposed 5 hours daily to 25,000 ft. (Baro. Press. 282 mm Hg, pO_2 59 mm Hg) for 2 to 6 weeks, exercise tolerance was greatly reduced and foci of mycardial necrosis and fibrosis were often found in the left periventricular apical myocardium. Repeated bleeding during 6 weeks at 25,000 ft. prevented polycythemia, a reduction in exercise tolerance, and minimized pathologic changes. After discontinuing the exposures to 25,000 ft., the apical lesions persisted, but exercise tolerance gradually returned to normal.

The value of cold-acclimation in increasing tolerance to other stresses such as exercise or hypoxia has been in dispute. We have found evidence that the mortality of cold-acclimated rats exposed to 5.4% O_2 (32,000 ft. equivalent) was much greater than in unacclimated rats at room temperature. At higher O_2 concentrations, such as 6.6 or 8.3% O_2 (28,000 and 23,000 ft. equivalencies), mortality was the same in both groups, but cold-acclimated rats developed significantly higher concentrations of plasma corticosterone and serum glutamic oxalacetic and pyruvic transaminses, aldolase, lactic dehydrogenase and creatine phosphokinase than unacclimated rats. Also cold-acclimated rats exposed to 6.6% O_2 showed a significant increase in incidence of fatty changes in striated muscle, more marked depletion of liver glycogen and greater elevations in serum urea nitrogen than unacclimated rats.

Significance to NIAMD Research:

Our findings show that severe polycythemia, in the absence of other tissue abnormalities, produced by exposure to moderate hypoxia or by cobalt administration, does not adversely affect exercise performance at sea level. However, polycythemia produced by severe hypoxia in the presence of focal myocardial lesions markedly reduces exercise performance. It is apparent that severe hypoxia is the principle factor responsible for the poor exercise performance and for the development of myocardial lesions, and that cardiac overloading caused by polycythemia is only an important secondary factor.

We have observed negative cross adaptation between cold-acclimation and hypoxia at room temperature. Increased corticosterone in the plasma and elevated activities of glycolytic and oxidative enzymes in the serum, suggests that the cold-acclimated rats were under more severe stress. The persistence of less severe hypoxic hypothermia in the cold-acclimated rats indicated that the increased metabolic rate, induced during exposure to cold, may account for the poor hypoxic tolerance.

Proposed Course of Project:

Studies will be conducted on deacclimation after cold-acclimation to determine the duration and extent of the deleterious effects of the cold. Studies will be conducted on the effects of exposure of rabbits to severe cold on tolerance and on the mechanism involved in the production of tissue changes and serum enzyme alterations. Investigations on the possible significance of hypoxia as a factor in the production of arteriosclerosis will be continued.

Publications:

Altland, P. D., Highman, B., and Dieter, M. P.: Reduced hypoxic tolerance of cold-acclimated rats: Serum enzyme and tissue changes. Am. J. Physiol. (in press).

REPORTED IN PRESS - 1971

Altland, P. D., Brubach, H. F., Parker, M. G., Dieter, M. P., and Murayama, M.: Effects of smoke on tolerance of rats to hypoxia. J. Appl. Physiol. 30: 352-357, March 1971.

Altland, P. D., and Highman, B. (with technical assistance of M. G. Parker): Effects of polycythemia and altitude hypoxia on rat heart and exercise tolerance. Am. J. Physiol. 221: 388-393, August 1971.

Dieter, M. P., and Breitenbach, R. P.: Vitamin C in lymphoid organs of rats and cockerels treated with corticosterone or testosterone. Proc. Soc. Exp. Biol. Med. 137: 341-346, May 1971.

Serial No. NIAMD-LPB-20
1. Physical Biology
2. Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Physico-chemical properties of biological surfaces and related systems.

Previous Serial Number: NIAMD-LPB-21

Principal Investigator: N. L. Gershfeld

Other Investigators: None

Cooperating Units: None

Man Years (July 1971 through June 1972)

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

(a) To measure the intermolecular energies of condensed lipid systems.

(b) To evaluate the influence of lipid structure on the properties of cellular surfaces.

Methods Employed:

General techniques of surface chemistry have been adapted and refined to measure intermolecular energies at fluid interfaces. Differential scanning calorimetry has been applied to evaluate the energetics of lipid interactions.

Major Findings:

The properties of lipid mixtures in monolayers on water follow the same general rules as hydrocarbon mixtures in bulk solution; they form regular solutions. One can predict, qualitatively, the mixing behavior of lipids given the physical state of the pure components. This analysis has been extended to the liquid-crystalline state of lecithins; in this system the mixtures are also dominated by interactions in the hydrocarbon region of the bilayer structure.

An exception has been found with cholesterol-lecithin mixtures where an intense interaction between the polar moieties leads to complex formation in anhydrous mixtures. When lecithin and cholesterol are suspended in water, the polar group interaction is decreased, as expected, and the interactions between the hydrocarbon moieties (the fatty acid chains of lecithin and the fused ring structure of cholesterol) then dominate the mixing of the two components. Cholesterol-lecithin mixtures in water follow the general behavior of these mixtures observed in monolayers, especially with respect to the correlation with the physical state of acyl moiety in lecithin.

Significance to NIAMD Research:

A set of operational principles for predicting the properties of lipid mixtures is being developed to evaluate the role of lipids in membrane function.

Proposed Course of Project:

The monolayer studies will continue with examination of parameters (e.g. hydrocarbon chain length, shape, etc.) controlling phase transitions in one component and multicomponent systems. Thermodynamic analysis of the liquid-crystal system will be developed to examine the influence of the polar groups in the cholesterol-phospholipid interaction.

Publications:

Gershfeld, N. L., and Muramatsu, M.: The interaction between steroid hormones and lipid monolayers on water. J. Gen. Physiol. 58: 650-666, 1971.

Gershfeld, N. L.: Film balance and the evaluation of intermolecular energies in monolayers. in "Techniques of Surface and Colloid Chemistry and Physics. ed. R. J. Good, R. R. Stromberg, and R. L. Patrick. Marcel Dekker, Inc., N. Y., 1972, pp. 1-39.

Pagano, R. E., and Gershfeld, N. L.: A millidyne film balance for measuring intermolecular energies in lipid films. J. Colloid Interface Sci. (in press).

Gershfeld, N. L., and Pagano, R. E.: Physical chemistry of lipid films at the air-water interface. I. Intermolecular energies in single component lipid films. J. Phys. Chem. (in press).

Pagano, R. E., and Gershfeld, N. L.: Physical chemistry of lipid films at the air-water interface. II. Binary lipid mixtures. The principles governing miscibility of lipids in surfaces. J. Phys. Chem. (in press).

Gershfeld, N. L., and Pagano, R. E.: Physical chemistry of lipid films at the air-water interface. III. The condensing effect of cholesterol. A critical examination of mixed film studies. J. Phys. Chem. (in press)

Serial No. NIAMD-LPB-21
1. Physical Biology
2. Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies of metabolic activity in microorganisms.

Previous Serial Number: 22

Principal Investigator: Ellis S. Kempner

Other Investigators: Jay H. Miller

Cooperating Units: None

Man Years (July 1971 through June 1972)

Total:	2
Professional:	2
Other:	0

Project Description:

Objectives:

An understanding of the control of metabolic activity in unicellular organisms.

Methods Employed:

Culture of microorganisms in the presence of radioactive or other compounds; chemical and physical fractionation of the cellular materials by ultracentrifugation, chromatography and chemical extraction. Microscopic examination of cells by time-lapse video micrography.

Major Findings:

Studies of the protozoan, Euglena gracilis, have been continued.

The tentative identification of mannitol in acid soluble extracts of Euglena gracilis was confirmed by paper chromatography, reactions with $AgNO_3$, benzidine and barium hydroxide, and by NMR Spectroscopy. Optical rotation showed it to be D-mannitol. Experiments with Euglena uniformly labelled with ^{14}C revealed mannitol to be the single largest component in the pool of metabolic intermediates. Smaller amounts of glycerol were also identified with the same techniques. No free sugars could be detected in the cell extracts.

Although exogenous mannitol is not incorporated by Euglena, tracer quantities of glucose and fructose are rapidly absorbed and converted to mannitol. The presence of both mannitol dehydrogenase and mannitol-1-phosphate dehydrogenase was established and the enzymes briefly characterized. These data indicated that the two different mannitol pathways known in bacteria are both present in the protozoan Euglena.

Two separate mechanisms of movement occur in Euglena. Locomotion is achieved by a beating flagellum, while changes in shape, called "metaboly" are independant of the flagellum. It was shown that the "metaboly" process occurs normally and continuously; it is uniquely a change in shape with no alteration in cellular volume. Further studies have shown that subcellular particles in Euglena stratified by centrifugation return to the normal state because of metaboly: the contortion of cell shape in metaboly disrupts the ordered stratification so that the organelles return to their normal random distribution in the cytoplasm. Time lapse video micrography was utilized to follow metaboly in stratified and control Euglena cells. No quantitative differences were observed. Therefore the recovery of Euglena from stratification is due to a normal activity of the cells; this activity is not diminished by the trauma of stratification, even though other general biochemical processes are temporarily inhibited.

Significance to NIAMD Research:

This work is aiding the development of a system for studying differentiated cellular structure in Euglena gracilis with the technical ease usual with bacteria. Using this system, new insight into the structure and function of cells is being gained.

Proposed Course of Project:

To study the metabolic relationships of synthetic processes in Euglena to the highly organized cellular structure which has already been elucidated.

Serial No. NIAMD-LPB-22
1. Physical Biology
2. Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Investigation of the macromolecular organization of living matter.

Previous Serial Number: NIAMD-LPB-23

Principal Investigator: L. W. Labaw

Other Investigators: None

Cooperating Units: None

Man Years (July 1971 through June 1972)

Total:	1.3
Professional:	1
Other:	0.3

Project Description:

Objectives:

To gain information about the macromolecules that are essential constituents of living matter and to see how they are arranged in the structures they form. To study certain of these macromolecules, such as viruses and other proteins, in purified form after isolation from the living material. To improve the resolution of the electron microscope and to interpret the way in which images are formed near its limit of resolution.

Methods Employed:

The electron microscopy of microorganisms, cells and tissues in suspension or thinly sectioned. The physicochemical characterization of macromolecular components isolated from such material using electron microscope, x-ray diffraction, and similar established techniques.

Major Findings:

Dr. Bror Strandberg of the University of Uppsala is attempting to extend x-ray crystallographic analyses, which have been used so successfully to give the three dimensional structure of many protein molecules, to viruses. His initial project is an x-ray study of the smallest virus known, the satellite tobacco necrosis virus (STNV). At his request, I have extended my previous (1959) electron microscopic study of the STNV crystal for

possible information about the internal structure of the virus. Preliminary results demonstrate the nucleic acid to be contained in a central sphere 108 Å in diameter. This agrees with a physical chemical study showing the nucleic acid to be about 20% of the total volume. The EM views through the crystal in the various crystallographic directions favor icosohedral symmetry of the protein subunits in the shell surrounding the nucleic acid, rather than the octahedral symmetry that the x-ray data seemed initially to suggest.

Significance to NIAMD Research:

The use of the electron microscope to determine the crystal structure of protein crystals is particularly important when applied to crystals which cannot be grown large enough for a more complete structure study by x-rays.

With increased emphasis on fine structure in electron microscopy, it is pertinent to examine the mechanism of image formation to define the conditions under which one can be assured that observed periodicities on images in the electron microscope have a direct spatial relationship with the object being examined when one is operating near the resolution limit of the instrument.

Proposed Course of Project:

To investigate other protein crystals in the electron microscope using replica, negative staining, and direct transmission techniques to determine their structures.

Honors and Awards:

Appointed to the editorial board of the J. Ultrastructure Research, Nov. 8, 1971.

Publications:

Davies, D. R., Sarma, V. R., Labaw, L. W., Silverton, W. W., and Terry, W. D.: Three-dimensional structure of immunoglobulins. Progress in Immunology, Academic Press, Inc., New York and London, 1971 (pages 25-32).

Labaw, L. W., and Davies, D. R.: The molecular outline of human γG1 immunoglobulin from an EM study of crystals. J. Ultrastruct. Res. (in press).

REPORTED IN PRESS - 1971

De Nayer, P., and Labaw, L. W.: Identification of large size polyribosomes in bovine thyroid glands. Endocrinology 88: 783-786, 1971.

Labaw. L. W., and Davies, D. R.: An electron microscopic study of human γG1 immunoglobulin crystals: Preliminary results. J. Biol. Chem. 246: 3760-3762, 1971.

ANNUAL REPORT SUMMARY

LABORATORY OF BIOPHYSICAL CHEMISTRY

The chief interest of this laboratory has been the study of the relationship between structure and function of biological macromolecules, especially those proteins involved in muscular contraction, blood coagulation, and in carrying oxygen. Advantage is also taken when pathological conditions offer insight into the problem under investigation. Special attention is paid to the interaction of these proteins within their environments, such as water, certain ions, and ATP.

MUSCLE: The Section on Bioenergetics, in collaboration with Dr. Witold Drabikowski of the Nencki Institute of Experimental Biology in Warsaw, has been studying the binding of tropomyosin and troponin to filamentous actin. In collaboration with Dr. Ute Stewart of Wurzburg, Germany, cells of smooth muscle have been prepared, and their physical and enzymatic response to excitatory reagents has been examined in the absence and presence of a specific antibody. (Kominz).

FIBRINOGEN: The role of the carbohydrate chains of various fibrinogens has been studied by means of labeling the terminal sialic acid residues with tritiated borohydride after selective periodate oxidation. (Laki, Mulhern).

High resolution NMR studies are also in progress to study the binding of co-fibrins (fibrinopeptides A and B) to pure human thrombin. (Laki, Yeh).

Newer and more active preparations of CRPP (Cortisone Released Protease Preparations) when incubated with fibrinogen act to release a single large peptide (B+) accompanied by slow coagulation. The yield is 3%. However, the CRPP-treated fibrinogen now responds in a different manner than previously reported to the action of thrombin. Coagulation still takes place but the supernate now contains different peptides than the normally expected A and B indicating that the CRPP has caused structural changes in the fibrinogen molecule making it more susceptible to thrombin action. (Gladner).

HEMOGLOBIN: A continued effort is being made to rationalize all the available hemoglobin data in terms of a realistic model. The basic features of the model Dr. Saroff proposed previously still seem sound. Chemical studies on possible buried and labile groups in hemoglobin are under way. (Saroff).

Studies of cooperative ligand binding in hemoglobin and the mode of aggregation and gelation of deoxyhemoglobin S are also being carried out. (Minton).

SERUM ALBUMIN: An effort is being made to crystallize human serum albumin suitable for X-ray crystallography. (Simpson).

PEPTIDES: The usefulness of the cyanate method for quantitating end-group analysis of proteins and peptides is explored (Irreverre and Saroff), and the preparation and synthesis of amino acids related to cystathionine is being continued. (Irreverre).

WATER: An extensive study is in progress to investigate the structure of water by paying special attention to the refractive properties of pure liquids and crystalline solids, and the structure and dielectric properties of liquid water. (Minton).

ATP: The energy source for muscular contraction is the cleavage of ATP. Quantum mechanical calculations using the so-called CNDO/2 method revealed that a significant part of the energy stored in polyphosphates may be accounted for by the small nuclear rearrangement which occurs when phosphates form polyphosphates. It appears then that the contractile protein to which ATP is attached magnifies the nuclear rearrangement of the ATP molecule when it decomposes to a "visible" shape change. (Alving, Laki).

Serial No. NIAMD LBC-1
1. Biophysical Chemistry
2. Bioenergetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Basic mechanisms of muscular contraction.

Previous Serial Number: LBC-2

Principal Investigator: Dr. D. R. Kominz

Other Investigators: Dr. Remedios Avena (through August 1971)
Dr. Ryoko Tsukui, Visiting Associate, Univ. of Tokyo
Mr. Clarence Israel

Outside Collaborators: Dr. Witold Drabikowski, Nencki Institute, Warsaw, Poland, Dr. Ute Stewart, Wurzburg University, Wurzburg Germany, Dr. Seth Goldstein, BEIB, DRS, Dr. Carl Mattern, NIAID

Man Years
Total: 3.5
Professional: 2.0
Other 1.5

Project Description:

Objectives: To advance the understanding of muscular contraction and its regulation.

Methods: In general, studying the enzymatic activity and physical properties of muscle model systems, such as purified contractile proteins, myofibril suspensions, smooth muscle cell suspensions, and glycerinated muscle fibers. Specifically, 1) studying the binding of ^{125}I-labelled tropomyosin or troponin to F-actin or desensitized myofibrils; 2) employing the Celloscope particle counter and Intertechnique Multichannel Analyzer to study length and volume changes of myofibrils and isolated smooth muscle cells; 3) employing a helium-neon laser with fiber-optics scanner to monitor sarcomere spacing and order within glycerinated fibers developing tension; 4) using a combination spectrophotometer-pH stat to measure ATPase activity and turbidity changes simultaneously in myofibril preparations; 5) employing SDS gel electrophoresis and other methods of characterization as needed.

Major Findings: 1. Tropomyosin and troponin bind independently to F-actin. Troponin-binding increases as the ionic strength is lowered from 0.12 to 0.02 ionic strength, and remains strong at very low ionic strength. Tropomyosin-binding is constant as the ionic strength is lowered, until it starts to drop at 0.02 ionic strength and becomes negative below 0.01 ionic strength.

The presence of tropomyosin does not affect the binding of troponin. (Drabikowski, Tsukui and Kominz)

2. Suspensions of isolated smooth muscle cells may be prepared by collagenase digestion of chicken gizzard slices. Serotonin and Norepinephrine cause slight contraction of these cells with no accompanying volume change; ATP causes a large contraction accompanied by a decrease in volume. This may be contrasted to myofibril suspensions where contraction had been found to be accompanied by a volume increase. (Stewart and Kominz)

3. Binding of specific antibody could be measured as increased volume of both myofibrils and of smooth muscle cells. At a 4/1 ratio of antibody to antigen, contraction of both myofibrils and smooth muscle cells was nearly completely prevented. However, at this ratio of antibody to antigen, myofibrillar ATPase activity was only reduced one-third, while the EGTA - sensitivity was also reduced one-third. (Stewart and Kominz)

4. In aged glycerinated rabbit psoas fibers, there is a direct correlation between development of tension and loss of the interference pattern which arises from the regular sarcomere spacing (Tsukui and Kominz). Electron microscopic examination of fibers which have lost the interference pattern reveals distorted structures with rest-length fibers alongside highly-contracted ones, suggesting that contraction in one region of such fibers is compensated by stretch in another region. (Mattern)

Projects Proposed: 1. Improved methods will be sought for obtaining intact and homogeneous preparations of smooth muscle cells or myofibrils, uncontaminated with mitochondria and other small particulate matter. In collaboration with Dr. Stewart, new antibody preparations will be examined.

2. A continuing goal is the simultaneous monitoring on glycerinated fibers of sarcomere length, tension, and ATPase activity. To this end, a number of laboratories in England and Germany will be visited this summer to observe instrumentation and preparative techniques. In collaboration with Dr. Seth Goldstein, BEIB, DRS, appropriate instrumentation will be designed and fabricated.

Significance to Bio-Medical Research and the Programs of the Institute:

Living systems are not in equilibrium, but either approaching or in steady state arising from coupled energetic processes. Muscle is a well-studied system in which the nature of the coupling between the energy-supplying process (ATPase) and the energy-utilizing process (tension or shortening) remains elusive. With a few exceptions, attempted reversal appears to produce an uncoupling of the two processes. It is hoped that new approaches may throw light on the source of the uncoupling, whether it be an artefact of the preparative or measuring procedures, a special attribute of muscle systems or a more general phenomenon.

Honors and Awards: None

Publications:

Avena, R. M. and Bowen, W. J.: Adenosine triphosphate inhibition in myosin adenosine triphosphate systems. J. Biol. Chem. 7: 2265-2270, 1971.

Kominz, D. R.: Role of swelling in muscle contraction. J. Theor. Biol. 31: 255-267, 1971.

Kominz, D. R.: The Regulatory ATP-Binding Site of Myosin. In Laki, K. (Ed.): Contractile Proteins and Muscle, New York, N. Y. Marcel Dekker, 1971, pp. 253-261.

Bowen, W. J. (deceased) and Mandelkern, L.: Glycerinated muscle fibers: relation between isometric tension and adenosine triphosphate hydrolysis. Science 173: 239-240, 1971.

Bowen, W. J. (deceased): Myosin ATPase and IPTase. In Laki, K. (Ed.): Contractile Proteins and Muscle, New York, N. Y. Marcel Dekker, 1971, pp. 219-251.

Serial No. NIAMD LBC-2
1. Biophysical Chemistry
2. Macromolecules
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The elucidation of the structure and interactions of biologically important macromolecules.

Previous Serial Number: LBC-7

Principal Investigator: H. A. Saroff

Other Investigators: Dr. R. B. Simpson, E. R. Mitchell and H. L. Wolff

Cooperating Units: Dr. Arthur Spector, Univ. of Iowa Clinical Research Center, Iowa City, Iowa; Dr. J. Preer, Federal City College, Washington, D. C., K. John, NHI

Man Years:
Total: $4\frac{1}{2}$
Professional: 4
Other: $\frac{1}{2}$

Project Description:

Objectives: The main objective of this laboratory is an elucidation of the structure of naturally occurring macromolecules based on the methods of physical chemistry. Macromolecules are studied primarily in solution and the emphasis is on the accumulation of data on the state of the molecules with respect to environment in solution. Gross features of structure as well as fine detail, may be observed in the study of macromolecules in solution.

Methods: At present, we are concerned with methods of observing macromolecules in solution. In our studies aimed at finer details of protein structure, the methods used involve the measurements of the binding of small molecules or ions to specific sites on the protein molecule, nuclear magnetic resonance (on model compounds, so far), optical rotatory dispersion measurements and other spectral techniques. For other properties of the protein molecules, we employ the ultracentrifugal, light scattering, electrophoretic and diffusion techniques.

Major Findings and Proposed Course of Studies: The anion 2, 3-Diphosphoglyceric acid (DPG) is an important factor in the action of hemoglobin. We have studied the binding of both H^+ and Na^+ to this compound and have analyzed the data in terms of the binding of cations to a cluster of negative charges.

The action of hemoglobin is being studied with emphasis on its equilibrium properties. Three aspects of the properties of hemoglobin are being studied:

1. Aggregation properties as applied to Lamprey hemoglobin.

2. The role of the SH group in the function of hemoglobin.

3. The energetics of the hemoglobin reactions.

Studies are being continued on the use of computational models for the binding of ions to clusters of ionizable groups in model compounds. (Preer, Saroff).

Attempts to crystallize serum albumin and a mercury derivative suitable for X-ray crystallography continue. (Simpson)

With Arthur Spector of the University of Iowa, and Kathryn John of the National Heart and Lung Institute, it was shown that the data on aqueous solutions of fatty acids can be explained without resorting to a hypothesis of dimerization of the acids or their anions.

Viscosities of hemoglobin solutions are being measured with a view to elucidating the difference in aggregation between normal and sickle cell hemoglobin. (Simpson, Minton)

Significance to Biomedical Research and the Programs of the Institute:

Our emphasis is on the relationship between chemical properties and the structure of macromolecules. Once these properties are properly related, the attack on the relationship between structure and function can proceed on a solid basis. The proteins and macromolecules studied are either enzymes or those of interest in biological systems. Our systems are highly controlled with reference to the conditions of the protein environment and the bio-medical significance of our findings derive from extropolation to the biological system.

Honors and Awards: None

Publications:

Saroff, H. A.: Interpretation of the differential titration data of des-(His146β) human hemoglobin. J. Physiol. Chem. & Physics. (1972) In press.

Saroff, H. A. and Yap, W. T.: The action of hemoglobin. Cooperative effects in tetrameric proteins. Biopolymers, (1972) In press.

Saroff, H. A. and Minton, Allen, P.: The Hill plot and the energy of interaction in hemoglobin. Science 175, 1253-1255, 1972.

Saroff, H. A.: Speculations on the origins of life and the mechanism of cancer. Perspect. Biol. Med. 15, No. 2 Winter, p. 307, 1972

Saroff, H. A.: The action of hemoglobin. Cooperative and Bohr effects. J. of Phys. Chem. 1972. In press.

Serial No. NIAMD LBC-3
1. Biophysical Chemistry
2. Physical Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Biochemical studies on physiologically important micro and macromolecules in living systems.

Previous Serial No.: NIAMD LBC-1

Principal Investigator: Filadelfo Irreverre

Technical Assistance: Mr. Edwin Wilson

Cooperating Units: Dr. J. L. Hartwell, DD, NCI

Man Years
Total: 2
Professional: 2

Project Description:

Objectives: In view of the complexity of interacting macro and micromolecules in living systems, it is of interest to direct studies on (1) the nature of some of the unknown nitrogenous micromolecules, and (2) structural features of some proteins, such as the influence of various residues with neighboring groups.

Methods: The usual techniques in chemistry and biochemistry have been employed, such as paper and column chromatography, automated amino acid analysis, thin layer chromatography, gel separation and filtration, paper and gel electrophoresis, UV, infrared and mass spectrometry, NMR, polarimetry, ultracentrifugation, radioisotopic techniques and enzymatic analysis. When necessary, development of new techniques of isolation, detection or micro-assay has been undertaken.

Major Findings: With Drug Development Branch, NCI furnishing the materials, we have examined various plant extracts looking for the presence of unusual amino acids. From over a dozen plant extracts examined to date, we have found some unidentified ninhydrin-positive compounds which are present only in small amounts, too minute for practical isolation. We are continuing this screening work with the hope of uncovering other unknown amino acids present in fairly large amounts to warrant isolation.

As a follow-up of our past work on homocystinuria and cystathioninuria, we have synthesized some S-derivatives of L-cysteine, such as S-(carboxymethyl)-L-cysteine, S(1-2-dicarboxyethyl)-L-cysteine and S-(β-carboxy-n-propyl)-

L-cysteine and the corresponding S-derivatives of L-homocysteine using published methods. These compounds have been recently found in human urines by Japanese workers. The synthetic compounds are being tested as competitive inhibitors in some of the sulfur enzyme systems such as cystathionase, cystathionine synthase and methionine-activating enzyme.

We are studying some aspects of the nature of the "buried" SH groups of hemoglobin and other proteins. (Irreverre, Saroff).

Significance to Bio-Medical Research and the Program of the Institute: Studies of the structures of newly isolated proteins, new amino acids and metabolites contribute to the ever expanding fabric of knowledge in living systems including man.

Proposed course of project: Biochemical studies of physiologically interesting micro and macromolecules in living systems will continue. Special emphasis will be given to studies on the nature of the "buried" SH groups of hemoglobin and other proteins.

Honors and Awards: None.

Publications:

1. Bodwell, C. E., Irreverre, F. and Hornstein, I.: Tropomyosin B from the African civet, Civettictis civetta. Comp. Biochem. Physiol. 40B, 571, 1971.

Serial No. NIAMD LBC-4
1. Biophysical Chemistry
2. Macromolecules
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Intermolecular interactions in physical biochemistry.

Previous Serial No.: NIAMD LBC-8

Principal Investigator: Allen P. Minton

Man Years:
Total: 1
Professional: 1

Project Description:

Objectives: During the past year, two kinds of intermolecular interactions have been investigated: 1) the interaction between water molecules in the liquid and solid phase, and the influence thereof upon optical and dielectric properties; and 2) the interaction between molecules of hemoglobin in solutions of normal and sickling species leading to "gelling" and subsequent sickling of erythrocytes.

Methods: The primary approach until recently has been theoretical, but preparations are currently underway for characterization of the aggregating properties of hemoglobin by means of high speed membrane osmometry.

Major Findings and Proposed Course of Studies:
A) Water: A structural model has been developed to account for anomalous aspects of the dielectric relaxation of liquid water. The effect of time-varying and constant anisotropic local fields upon the refractive properties of liquid water and ice has been investigated and a theory of the temperature and pressure dependence of the refractive index developed.

B) Hemoglobin: A theory has been developed to account for the gelling properties of binary mixtures of any two of the three species, A, S and C_H. Relations between the results of osmotic pressure experiments and aggregation properties have been derived and it is hoped that accurate measurement of the second osmotic virial coefficient under a variety of conditions (to be undertaken in this laboratory) will allow us to evaluate aggregation constants characterizing the systems studied.

Significance to Biomedical Research and the Programs of the Institute: An understanding of the relation between structure and properties of liquid water is fundamental to any understanding of the behavior of aqueous biological systems. A characterization and understanding of the aggregating properties of sickling and normal hemoglobins is necessary to an understanding of the molecular basis of sickle cell anemia.

Honors and Awards: None

Publications:

Minton, Allen P.: Relations between crystal structure, molecular electronic polarizability and refractive properties in Ice I. J. Phys. Chem. 76: 886, 1972

Minton, Allen P.: A structural model for the dielectric relaxation of liquid water. Nature Physical Science 234: 165-168, 1971.

Reisler, E., Eisenberg, H., and Minton, Allen P.: The temperature and density dependence of the refractive index of pure liquids. Faraday Transactions. In press.

Minton, Allen P.: Comments on extensions of the allosteric model for hemoglobin. Nature New Biology 232: 145 1971.

Serial No. NIAMD LBC-5
1. Biophysical Chemistry
2. Physical Biochemistry
Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Proteins, enzymes and peptides involved in blood coagulation and inflammation.

Previous Serial No.: NIAMD LBC-4

Principal Investigator: Jules A. Gladner

Other Investigators: Dr. Albert J. Osbahr
Mrs. Patricia A. Murtaugh
Mr. Raymond Jacob, former Guest Worker

Cooperating Units: Dr. John C. Houck, Children's Hospital, Wash., DC and the George Washington University Medical School, Wash., DC; Dr. John Halver, Chief, Fish Nutrition Laboratory, Fish and Wildlife Service, Dept. of Interior, Cooke, Washington; and Dr. Claude B. Klee, NIAMD-LBP.

Man Years:
Total: 4
Professional: 4

Project Description:

Objectives: Involved in investigation are two systems vital to life processes. These systems, which appear unrelated, do involve similar, but not identical, mechanisms of the protein-protein interaction type. The first is a study of the thrombin-catalyzed conversion of fibrinogen to fibrin and all of its ramifications. This now includes the further steps of fibrin cross-linking. The second of these investigations is focused on the proteolytic enzymes which appear to play a major role in the biochemistry of the inflammatory response. Here, as in the first case, protein-protein interactions of the "limited protease" type take place. The objectives were extended to examine the effect of some of the latter enzymes on the former system to ascertain the possibility of these effects occurring physiologically.

Methods: Methods employed consisted of protein-enzyme fractionation, purification, characterization and modification. Where needed, methods of physical biochemistry and analytical chemistry were utilized, such as but not limited to, SDS gel electrophoresis and automatic amino acid analysis. Physiological techniques were utilized to examine the biological activity of various peptides. As applicable, new techniques were developed.

Major Findings: The conversion of fibrinogen to fibrin and related processes. One of the processes examined was the effect proteolytic enzymes produced via the inflammatory processes may have on the thrombin catalyzed conversion of fibrinogen to fibrin. We have shown in the past that the injection of anti-inflammatory agents, such as cortisone, into rats induces the formation or release of proteases in rat skin soluble in 0.15M NaCl. These "Cortisone Released Protease Preparations" (CRPP) were shown to contain at least 3 protease activities. One of these was chymotryptic in nature and rapidly hydrolyzed the nona-peptide, bradykinin, an agent found under conditions of physiological stress, such as inflammation, infections, burns, etc. Since this enzyme was limited in its ability to hydrolyze all chymotryptic type bonds, it was compared as to its action on fibrinogen to thrombin, an enzyme of known bond and protein specificity. In initial experiments utilizing CRPP on purified bovine fibrinogen, a slow coagulation was observed with a yield of only 2-3%. This was due to release of a single large peptide containing within it the moiety of peptide B, one of the two peptides released from fibrinogen by thrombin action. Following removal of unreacted fibrinogen, it could be shown that addition of thrombin to this fibrinogen brought about a normal clotting with the subsequent release of the A and B peptides. In our later investigations, we have been able to use CRPP preparations of greater potency. Again there ensues a slow "clotting" when the enzyme preparation is added to purified fibrinogen. Upon recovery of the fibrinogen and the subsequent addition of thrombin, we obtain clotted material of a more viscous type. The supernate of this material upon examination for the normal A and B peptides expected via the thrombin action does not appear to yield these same peptides. Judging from the appearance of the fibrin clots and tentative characterization of the non-protein nitrogen materials (peptides), it would appear that the CRPP preparations have hydrolyzed a small number of other bonds in the fibrinogen molecule; the number and nature of these are under investigation. Thus, the clotting via the thrombin pathway has been affected and even altered, but not terminated. This is in contrast to the effect of other known proteases reacting on fibrinogen subsequent to the addition of thrombin. Both trypsin and chymotrypsin, when added to fibrinogen prior to addition of thrombin, bring about the almost immediate and complete cessation of clotting. It is also possible in our case that the CRPP action on fibrinogen may not only bring about the hydrolysis of a limited number of peptide bonds, but may so alter the fibrinogen molecule so that thrombin itself may hydrolyze other bonds than in the native molecule. Thus, an enzyme becomes a biological probe in efforts to examine the fine structure involved in the key reaction of blood coagulation, the conversion of fibrinogen to fibrin by thrombin. (Gladner, Murtaugh, Jacob, Houck).

Cross-linking of fibrin. Following the conversion of fibrinogen to ribrin by thrombin, another enzyme Factor XIII cross-links the fibrin via a transglutaminase action into an insoluble network which is actually the physiological clot. Most of the mechanism of action for this reaction has been elucidated for the higher species; i.e., human, bovine, etc. We have

shown that the fibrinogen-fibrin transformation appears to be the same for the lower species, such as frog. To date, the cross-linking of fibrin monomers of lower species has not been reported. To this end, we are investigating the cross-linking of various fibrins of the low species, especially salmon and lamprey eel. The latter is of extreme interest in that the lamprey fibrinogen yields only one peptide (B) via bovine thrombin and two peptides (A and B) via lamprey thrombin. These, in turn, are expected to yield different cross-linking patterns. Along with these fibrins, examination of chemically (via succinylation) and enzymatically (via CRPP) modified bovine fibrinogen and fibrin as to the effect of cross-linking by Factor XIII is also under study. These investigations are directed toward gaining insight as to the similarity of action throughout the species, as well as elucidation of the role that protein structure itself plays in cross-linking. (Murtaugh, Gladner).

Isolation of active site. The active site of the enzyme histidine, ammonia-lyase, has been isolated and characterized. Experiments utilizing radioactive tracers, enzymatic degradation, column chromotography, peptide mapping and amino acid analysis were employed. (Klee, Gladner).

Significance to biomedical research and the program of the Institute: Although a great deal is known concerning the protein-protein interactions involved in blood coagulation, very little is known concerning the biochemistry of anti-inflammatory drugs and inflammation. In this report, we have utilized materials from one source to investigate the other since physiologically they occur concurrently. At the same time we are studying "limited proteases", proteolytic enzymes, which, unlike digestive enzymes, play a very important role in performing very specific reactions. More of these types of enzymes are being reported and the more we know of them, the more we will be able to relate them to actual biological mechanisms.

Proposed course of project: It is anticipated that investigation will proceed along the general lines set forth in the bulk of this report. The CRPP enzymes will be further purified and absolute specificity studies will be carried out on a series of natural substrates.

Honors and Awards: None.

Publications: None.

Serial No. NIAMD LBC-6
1. Biophysical Chemistry
2. Physical Biochemistry
Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Topography of fibrinogen-thrombin interaction.

Previous Serial No.: None

Principal Investigator: K. Laki
Dr. Sally A. Mulhern

Cooperating Unit: Dr. Herman J. C. Yeh, A:LC, Vis. Associate

Technical Assistance: None

Man Years
Total: 2.0
Professional: 2.0

Project Description:

Objectives: The interaction of fibrinogen with thrombin is a vital process, the speed of which is adjusted during evolution to serve the survival of the animals. For the understanding of this delicately adjusted system, we need to understand the details of this enzyme-substrate combination. In this project, it is demonstrated that even without X-ray diffraction studies, a great deal can be learned from indirect experiments.

Methods: Methods applied in this project utilize the techniques of protein purification together with the use of tritium labelling, scintillation counters, paper and column chromatography, UV spectroscopy, high resolution NMR spectroscopy, and others.

Major Findings: Topography of the fibrinogen-thrombin interaction. Previously we reported the correlation between the clotting time of various fibrinogens by thrombin and the amino acid sequence of the peptides which were released in the clotting process. From these studies, it was concluded that the N-terminal portion of α-chains of the various fibrinogens are aligned along the thrombin molecule up to the arginine residue where the hydrolytic cleavage takes place. Since the amino acid sequence of thrombin is known, it can be concluded that in the enzyme-substrate combination, the N-terminal portion of the α-chain of fibrinogen (peptide A) attaches to the N-terminal portion of thrombin in a parallel beta conformation. A small portion of peptide A surrounding the arginine bond which is split is attached in a cross-beta position to the serine residue located in the active site of thrombin. From high resolution NMR studies, a chemical shift was

observed when human peptide A interacted with pure human thrombin. This supports the conclusion that indeed a number of residues far from the C-terminal arginine in peptide A interact with thrombin.

Carbohydrate content of fibrinogen. In order to gain further insight into the role of carbohydrate chains in fibrinogen, the chains were labelled by making the terminal sialic acid residues radioactive. For the labelling procedure, we selectively oxidized with periodate the sialic acid and then reduced it with tritiated borohydrate. Although the resulting labelled sialic acid is the seven-carbon variety, this alteration has no effect on the clotting of fibrinogen. Using these labelled fibrinogens after reducing the disulfide bonds and separating the chains on polyacrylamide gel electrophoresis in the presence of SDS, we were able to show that the three chains of bovine, porcine, rabbit and dog fibrinogen contained carbohydrate residues.

Significance to biomedical research and the program of the Institute: More and more instances of clotting defects due to abnormal fibrinogen are coming to light. In order to understand these genetically controlled defects, the detailed study of the fibrinogen-thrombin interaction is essential.

Proposed course of project: Effort is being made to further identify the locus on thrombin close to the N-terminal portion where mutation regulates the speed of thrombin action.

Honors and Awards: Member Dow Oxygenator Task Force for NHLI; Chairman, New York Academy of Sciences Conference on the Biological Role of the Clot Stabilizing Enzymes, New York City, November 18-19, 1971.

Publications:

1. Alving, R. E. and Laki, K.: Energy and Electron Orbitals in Adenosine Phosphates. J. Theor. Biol. 34: 199-214, 1972.

2. Laki, K.: Our Ancient Heritage in Blood Clotting and Some of Its Consequences. Proc. New York Acad. Sci. Conference on the Biological Role of the Clot Stabilizing Enzymes, Nov. 18-19, 1971.

3. Laki, K.: Clot Stabilization and Atherosclerosis. Proc. New York Acad. Sci. Conference on the Biological Role of the Clot Stabilizing Enzymes, Nov. 18-19, 1971.

4. Yancey, S. and Laki, K.: Transglutaminase and Tumor Growth. Proc. New York Acad. Sci. Conference on the Biological Role of the Clot Stabilizing Enzymes, Nov. 18-19, 1971.

5. Laki, K.: Hemostasis in the Animal Kingdom. Proc. Natl. Conference on Research Animals in Medicine, NHLI, Wash., D.C., Jan. 28-30, 1972.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The action of thrombin on fibrinogen

Previous Serial No.: NIAMD LBC-6

Principal Investigator: A. J. Osbahr

Cooperating Unit: Manual Morales, Cardiovascular Research Institute, San Francisco, California

Man Years
Total: 1
Professional: 1

Project Description:

Objectives: The chemical structure and the physiological role of the peptides released from fibrinogen was investigated. Modification of thrombin, fibrinogen and the peptides derived from them was studied to ascertain the site of enzymatic and physiological activity.

Methods: The methods applied to study these systems were those of structure determination and sequence analysis with the special application of techniques to evaluate homogeneity and purity of the systems under investigation. More specialized techniques of physiology were utilized when needed to study the biological role of the products of blood coagulation.

Major Findings: Frog fibrinogen was prepared and characterized on sephadex G-200. Two components were isolated and studied by ultracentrifugation techniques. Two peptides were released from the frog fibrinogen during clot formation. These peptides were separated on ion exchange chromatography and the purity ascertained by electrophoresis and chromatography. The analysis of the peptides was determined and indicated that the peptides differ appreciably in size and composition. Both peptides were shown to contain arginine C-terminal.

In order to study in the future the effect of reducing the negative charge on the physiological activity of the canine A peptide, the peptide was modified by methylation of its carboxyl groups to be tested for physiological potentiation of the bradykinin-induced contraction of rat uterus. The methylated peptide was separated on Dowex-50 by gradient elution. Additional modification of the canine A peptide will be carried out to enable us to ascertain the participation of functional groups in the

physiological activity of the potentiation of the bradykinin-induced contraction. These peptides are postulated as assisting in local hemostasis by vasoconstriction.

The canine A peptide when incubated with isolated purified myosin B resulted in a 40% increase in ATPase activity and a 200% increase in super-precipitation over and above control levels. We suggest the highly charged peptide interacts with the myosin B complex which enables increased binding of ATP to the actin-myosin complex and thereby results in an increased enzymatic activity. Modification of the negative charge on the canine A peptide may give us an insight into the mechanism involved in the vasoconstriction induced by the peptides.

Significance to biomedical research and the program of the Institute: Our studies indicate a possible relationship of these peptides from fibrinogen to blood pressure diseases and their effect on capillary pressures.

Modification of the blood clotting proteins and peptides lead us to a greater insight into the relationship between structure, physiological behavior and enzymatic activity.

Proposed course of project: Further modification and evaluation of the structure and physiological role of peptides from fibrinogen will be investigated, as well as a continuation of the structural effects of the enzyme thrombin and the mechanism of its action on fibrinogen.

Honors and Awards: None.

Publications: None.

ANNUAL REPORT SUMMARY

Laboratory of Molecular Biology

The work of the Laboratory of Molecular Biology is directed to an explanation of biological processes at the molecular level and to an understanding of the physical and chemical foundations of biological processes. Areas of investigation in the laboratory include study of the mechanisms of replication of DNA and of genetic recombination, studies of enzymes involved in recombination, characterization of mammalian and bacterial viruses by genetic analysis, studies of the molecular basis of viral properties, studies of the mechanism of protein synthesis, physical and chemical studies of the structure and properties of polynucleotides and nucleic acids, synthetic and spectroscopic investigation of polynucleotides, characterization of protein-DNA interaction in chromatin, investigation of protein structure by X-ray diffraction, and theoretical studies of nerve membrane characteristics. Major advances in several of these areas have been made by members of the Laboratory during the past year.

Mechanism of replication of lambda phage DNA.

The mechanism of replication of lambda phage DNA has been studied. Replicating molecules were isolated and their structure has been analyzed. Preliminary analysis indicates that at least a part of newly synthesized DNA strands are covalently attached to the parental strands. Functions of phage and bacterial genes for phage DNA replication have been studied. (Tomizawa, Sakakibara, Fuke).

Genetics and physiology of recombination deficiency of Escherichia coli.

Mutants which express temperature-sensitive recombination deficiency have been isolated, and genetic and physiological properties of these mutants were examined.

The ATP-dependent deoxyribonuclease of a temperature-sensitive recB mutant is heat-sensitive, indicating that the recB gene is a structural gene of the enzyme. Although the enzyme of a temperature-sensitive recC mutant is heat-stable, from the low enzyme activity of recBtsrecCts bacteria grown at the permissive temperature, the recC gene is argued to be another structural gene of the enzyme. Physiological study on recAts mutants is in progress (Tomizawa).

Functions involved in genetic recombination.

The physiological role of DNA ligase in E. coli is being studied. Several temperature-sensitive DNA ligase (lig) mutants have been isolated and mapped. These mutations, and a function (lop) which controls the expression of DNA ligase, all map in a tightly linked cluster near the chromosomal marker ctr. Another ligase mutation, originally isolated by C. Pauling in a genetically undefined E. coli strain, has been transduced into a well-defined strain in order to confirm that the defective

ligase is responsible for the failure of his strain to grow at high temperature. Since the transductants have the same property, DNA ligase is shown to be an essential enzyme for the growth of E. coli.

We have also shown that a defective DNA ligase, which does not reduce the growth rate of the cells, can nevertheless slow the joining of Okazaki fragments during DNA replication by at least tenfold. Thus the sealing of fragments must not be a rate-limiting step in DNA replication.

Further genetic studies will be greatly eased by the recent isolation of a viable lambda phage which carries the entire ligase region (M. Gellert, M. M. Gottesman, M. L. Hicks).

Studies of the molecular basis of biological control mechanisms in bacterial viruses.

1. Phage DNA from φ80 and φ80dhis (φ80 which carries the histidine genes from S. typhimurium) is being prepared and used in binding studies to the first enzyme in histidine biosynthesis. Preliminary data seems to indicate that the first enzyme binds specifically to his DNA. (M. Levinthal in collaboration with R. Goldberger, LCB).

2. Bacteriophage P1 directs the production of modification (methylase) and restriction (endonuclease) enzymes. It has been shown that P1 is itself a substrate for the P1 modification and restriction system. Two nonlysogenizing mutants of P1, c2 and c3, have been found to be deficient in the ability to modify their own DNA, and are therefore susceptible to P1 restriction. Furthermore, complementation studies and analyses of pseudo-revertants suggests that the non-lysogenizing characteristic is a consequence of cell death brought about by P1 restriction in the absence of P1 modification (J. Rosner).

3. Morphogenesis of bacteriophage heads has been studied by genetic analysis. Isolation and genetic mapping of a bacterial mutant (designated mop) and of phages capable of growing on it have led to the conclusion that morphogenesis of T4 phage heads is catalysed by a bacterial factor controlled by mop gene and that defect of this factor is compensated by mutation of gene 31 of phage. Since the bacterial mutant also restricts phage λ and φ80, the bacterial function may be involved generally in formation of phage heads (T. Takano).

4. Bacteriophage P1 can lysogenize bacteria and replicate as a plasmid synchronously with the cellular division cycle, without integration into the host chromosome. Experiments with a recently isolated bacterial mutant (lon ; cf. PNAS, 68, 1496 (1971), which is not lysogenized by P1, suggest that the product of lon gene acts as a repressor for P1 prophage and is needed for maintenance of synchronous replication of P1 prophage (T. Takano).

5. Suppressor mutations which partly replace the function of gene 32 (known to be required for genetic recombination and DNA replication) are under study in the T4 phage system. These suppressors delay the

replication of T4 DNA for several minutes, although finally a full quota of DNA is made. They may function by sparing the requirement for 32-protein early in replication (J. W. Little).

Studies of the oncogenic virus SV-40.

1. An endonuclease associated with SV40 virions has been partially characterized. This endonuclease makes multiple single strand nicks of superhelical SV40 DNA. The activity is associated with whole virions grown in a number of different animal cell lines. A number of temperature sensitive mutants has been screened, but none are defective for the activity even at the nonpermissive temperature. Attempts to dissociate the endonuclease from whole virions have been unsuccessful. (R. Saral and R. Martin in collaboration with H. Ozer, Wistar Foundation and W. Kidwell, NCI).

2. Characterization of a DNA-protein complex from SV40 virions has been undertaken. At high pH virions can be dissociated yielding a DNA-protein complex. Approximately 1% of the DNA in the complex is resistant to staphylococcal nuclease digestion. The remaining fragments are approximately 100 nucleotide pairs in length. Experiments are in progress to determine whether the protein is bound to the DNA at specific sites (P. McCann and R. Martin).

3. Further temperature sensitive mutants of SV40 are being isolated (J. Chou and R. Martin).

Polynucleotide studies.

1. Research continues into the forces leading to formation of ordered single strand structures of polynucleotides. Previous studies of polyriboadenylic acid (poly A) have been extended to low temperature; under these conditions the polymer is in a highly ordered form, and behaves like a nearly rigid rod. The dimensions of this rodlike molecule can be determined by light scattering, and appear to be consistent with observed repeat distances of ordered, helical, multi-strand polynucleotide structures. The effect of variation in solvent upon poly A conformation at higher temperatures has also been studied. The measurements confirm the conclusion of earlier experiments that the 'disordered' form of a single strand polynucleotide is in fact highly restricted with regard to the range of local conformations available to it, and that this restriction arises from limitations to freedom of rotation about the bonds of the polynucleotide backbone.

2. Precipitation of several polynucleotides has been studied as a function of pH. Poly A was found to precipitate in irregular plates upon freezing solutions at pH values at which it is normally soluble. The diffraction pattern of the plates suggests that the helix axis of the molecular structure (cf. Rich, Davies, Crick and Watson, J.M.B, 1961) is confined to the plane of the plate but is oriented randomly in that plane (S. Zimmerman).

3. We have synthesized poly 2-dimethylaminoadenylic acid and investigated properties of the homopolymer. It forms a protonated helical self-structure of much higher stability than that of poly A. The optical properties (UV, CD, TR) of the neutral, unprotonated polymer show marked temperature dependence, presumably as a result of single strand stacking. The temperature profiles of the different optical properties are quite different from each other, however, indicating that each property has a different sensitivity to structure. The usual assumption that fraction of ordered structure is a linear function of change in an optical property thus appears not to be valid for this polynucleotide (Ishikawa, Frazier, & Miles).

4. While it has been recognized for a number of years that different hydrogen bonding schemes of the bases might, in principle give rise to structurally isomeric helices, all of the known double helices have existed only in a single form, the Watson-Crick structure. We have recently demonstrated for the first time the formation of a two-stranded A·U helix with a non-Watson-Crick structure. Stereochemical control of the helical structure was achieved by introducing a dimethylamino substituent into the 2-position of poly A, thus restricting hydrogen bonding of poly U and poly BrU to the N_7 position of the adenine residues. The evidence suggests that the usual exclusive formation of Watson-Crick rather than Hoogsteen helices may be due to the high cooperativity of the interactions rather than to a large difference in energy between the two structures (Ishikawa, Howard, Frazier and Miles).

5. We have found that when poly rT and poly rA are mixed in a 1:1 ratio there is rapid formation of both two-stranded (A·T) and three-stranded (A·2T) helices. The latter species is metastable with respect to the two stranded helix under these conditions but can be converted to it only by a very slow displacement reaction (A·2T + A → 2A·T) requiring three or more days for completion. We have preliminary evidence that this phenomenon of formation of metastable three stranded helices in 1:1 mixtures is probably general for A·U interactions and believe that it is responsible for some of the unconclusive or erroneous determinations of polynucleotide stoichiometry which have been published (Howard, Frazier, and Miles).

Protein structure.

1. A more refined electron density map (at 6Å resolution) of the cryoglobulin DOB has been calculated. Some new features were observed, but the overall appearance of the molecule was in complete agreement with earlier work.

2. We have commenced a search for crystalline Fab fragments of these mouse myeloma proteins that are known to bind very tightly to small molecules. Crystals have been obtained from several proteins and one in particular, McPC 603, has yielded crystals suitable for a detailed X-ray diffraction analysis. (D. R. Davies, E. A. Padlan, V. R. Sarma, D. M. Segal, E. W. Silverton, in collaborstion with Drs. Potter and Rudikoff, NCI).

3. The acid protease from Rhizopus chinensis has been examined at low resolution (6Å). Data have now been collected to 3Å and heavy atom

positions are being refined prior to the calculation of an electron density map (G. H. Cohen, D. R. Davies, D. M. Segal, I. D. A. Swan.)

Structure of chromatin.

Chromatin is the complex of DNA and proteins isolated from the nuclei of eucaryotic organisms. It is believed to contain the information necessary for specifying cellular differentiation. Studies of the structure of chromatin have continued. Techniques have been developed for removing selectively the lysine-rich and slightly lysine-rich histones without causing rearrangement of the arginine-rich histones, which remain bound to DNA at their original binding sites. By selective digestion of the DNA of this partially stripped chromatin, it is possible to show that the arginine-rich histones are bound to guanine-cytosine rich DNA. This is the first demonstration of a unique natural binding site for a histone fraction, and may be of importance for understanding the relationship between structure and function on chromatin (R. Clark and G. Felsenfeld).

Protein synthesis.

Investigations have continued on the chemical and enzymatic properties of the two factors necessary for polypeptide chain elongation in mammals (EF1 and EF2, formerly aminoacyl transferase I and II).

EF2 and its inactive adenosine diphosphate ribosyl derivative, (ADPR-EF2) were found to consist of single polypeptide chains of about 1000 residues. The molecular weight calculated from the composition is 110,000, in close agreement with our previously reported value of 96,000. Each mole of enzyme contains 18 sulfhydryls and 2 disulfides.

EF1 has been resolved into non-identical components which do not differ markedly in enzymatic properties.

EF1 and EF2 appeared unable to interact with ribosomes at the same time, suggesting overlapping sites for the two factors (E. Maxwell, J. Collins, and E. Tudor).

Theoretical studies of nerve membranes.

1. The noise power spectrum from the open-close Hodgkin-Huxley kinetics of K^+ channels has been derived. The result does not correspond to the experimental 1/f spectrum. Hence the latter presumably originates in ion transport through channels which are already open (Hill and Chen).

2. Two theoretical models have been studied which might account for the observed delay in the appearance of a K^+ current when depolarization to around the Na^+ potential is preceded by a considerable hyperpolarization. (Hill and Chen).

3. The free energy (ΔG) and the activation free energies for the subunit conformational changes, presumably responsible for the opening of K^+

and Na^+ channels, can be deduced from experimental data of the Hodgkin-Huxley type. These free energies are functions of V (membrane potential). In particular, ΔG is a simple quadratic function of V. Most likely, the linear term is due to a change in net charge and the quadratic term to a change in a polarizability. (Hill and Chen).

4. We have studied the exact kinetics (via computer) of small cooperative Ising systems. We have investigated particularly the departure of such systems from internal equilibrium, as a function of time. (Paul and Hill).

5. Collaborative experimental work has been carried out on the biophysics of artificial black lipid membranes "decorated" with EIM (a protein which apparently forms channels for ion transport). (Blumenthal, in collaboration with Ehrenstein and Lecar, NINDS).

Thermodynamics.

1. Theoretical studies are in progress on the initiation of structure in simple models of biochemically reacting systems. (Blumenthal).

2. A statistical mechanical and thermodynamic study has been started on the binding of complementary monomers, and related molecules, onto polynucleotides (e.g., A + poly U). This supplements earlier experimental work of Miles, Ross, and their colleagues. (Hill).

3. The thermodynamic effects of exposing nucleic acid bases to water, have been determined by measurement of the solubility of adenine, cytosine and uracil in water and in organic solvents as a function of temperature. Transfer of a nucleic acid base from an organic environment into water is characterized by positive values for ΔH and for ΔS. (R. L. Scruggs, E. K. Achter, and P. D. Ross).

4. The heat changes occurring during metabolism of human blood platelets in the presence and absence of inducers and inhibitors of aggregation have been observed calorimetrically. Each inducer has a different effect on heat production of platelets. The heat increase produced by thrombin is especially large apparently because of increased platelet metabolic activity. (P. D. Ross in collaboration with A. P. Fletcher, American National Red Cross).

Serial No. NIAMD-LMB-1
1. Laboratory of Molecular Biology
2. Metabolic Enzymes
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Protein Synthesis in Mammals: Mechanisms and Metabolic Controls

Previous Serial Number: NIAMD-LMB-2

Principal Investigator: Elizabeth S. Maxwell

Other Investigators: James F. Collins
Elizabeth Tudor

Man Years
Total: 3
Professional: 3

Project Description:

Objectives: Our overall objective is to gain an understanding of the mechanisms and metabolic controls of protein synthesis in mammals. We hope to obtain a partial understanding of these processes through detailed knowledge of the soluble enzymes involved in polypeptide chain initiation, elongation and termination.

Methods Employed: All of the methods employed are either standard procedure or minor modifications thereof.

Major Findings: Investigations have continued on the chemical and enzymatic properties of the two factors necessary for polypeptide chain elongation in mammals (EF-1 and EF-2, formerly aminoacyl transferase I and II).

EF-2 and its inactive adenosine diphosphate ribosyl derivative, (ADPR-EF-2) were found to consist of single polypeptide chains of about 1000 residues. The two forms have the same amino acid composition and each has valine as its NH_2 terminal residue. The molecular weight calculated from the composition is 110,000, in close agreement with the value of 96,500 previously reported from this laboratory. Titration with ^{14}C p-chloromercuribenzoate indicated 18 sulfhydryls and 2 disulfides per mole of enzyme. Certain differences, indicative of conformational changes, were observed with EF-2 under varied conditions, as well as between EF-2 and ADPR-EF-2.

EF-1 has been resolved into multiple components by several techniques. Components of molecular weight higher than 170,000 break down during purification to active components of different electrophoretic mobilities and isoelectric points. No marked differences in enzymatic properties

were observed between the various components. The most highly purified fractions had all the activities expected from earlier studies with less pure preparations.

EF-1 and EF-2 appeared unable to interact with ribosomes at the same time, suggesting overlapping sites for the two factors.

Significance to NIAMD Research: The enzymatic mechanisms in protein synthesis in mammals and the metabolic control of these processes is of major interest to biology and biochemistry. Our emphasis so far is on enzymatic mechanisms. Hopefully, understanding of control will follow.

Proposed Course of Project: We intend to pursue our investigation of the various steps involved in the overall process of protein biosynthesis in mammalian cells. Our eventual aim is to have all the components necessary for the process purified in order to be able to make a specific protein in vitro and to study the control of that formation.

Further chemical characterization of EF-2 and its ADPR derivative are continuing, including the isolation of the tryptic peptide to which the ADPR is attached.

Studies on the purification of EF-1 are being completed. The interaction of EF-1 and EF-2 with the ribosomes is being studied by examining ribosomal proteins with which the factors may interact.

Studies on factors required for polypeptide termination in liver are beginning. We plan at first to use a model system for termination to locate such factors. Factors for initiation, elongation and termination will be required for the translation of a mammalian messenger RNA, such as a viral RNA, in vitro.

Serial No. NIAMD-LMB-2
1. Laboratory of Molecular Biology
2. Section on Metabolic Enzymes
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Enzymatic Joining of DNA Strands and its Role in Genetic Recombination

Previous Serial Number: NIAMD-LMB- 1

Principal Investigator: Martin Gellert

Other Investigators: John W. Little
Michael M. Gottesman
Minnie L. Hicks

Man Years

Total:	4
Professional:	3
Other:	1

Project Description:

Objectives: To understand the physiological role of DNA ligases; to study the biochemical basis of genetic recombination.

Methods Employed: Ultracentrifugation, standard enzymological and microbiological techniques.

Major Findings: 1) DNA ligase mutants of E. coli. (M. Gellert, M.M. Gottesman, M.L. Hicks).

a). Genetics: Using our previously isolated mutants, we have mapped the structural gene for DNA ligase (lig) and a neighboring locus (lop) which controls the synthesis of ligase. By a combination of bacterial mating and P1 transduction, both loci are placed near 46' on the standard E. coli map, the local order being recA - - - dapE ctr lop lig trzA aroC - - - - his. Of the two closest loci, ctr is a pleiotropic sugar-utilization function and trzA determines resistance to triazole.

By use of an F' episome which covers this region, we have shown that lig is recessive to lig^+, and that lop is cis-dominant to lop^+, as expected for a promoter or operator site.

b). Double mutants: Starting with our temperature-sensitive lig4 mutant, which has 1% of normal ligase activity at 42° but still grows well

at that temperature, we have introduced a number of other mutations related to DNA metabolism. A polA lig4 strain fails to grow at 42°, and recA lig4 or recB lig4 strains grow very poorly. All these strains grow well below 37°. There is a possible analogy here to the properties of polA strains (lacking polymerase I), since polA recA and polA recB combinations are known to be lethal. Why two non-lethal mutations should combine to produce a lethal effect is a problem under study.

c) Relation to phage functions: Some coliphages (e.g. T4, T7) code for their own ligase activity. While T4 mutants lacking ligase do not grow, the ligase function of T7 is normally dispensable; even T7 strains in which the ligase gene is deleted are able to grow on standard E. coli hosts. However, T7 lig does not grow in a ligase-defective host, which shows that the normal level of host ligase is sufficient to support T7 growth, but that a minimal level of ligase, either host-coded or phage-coded, is a requirement for T7 as well as T4.

Phage λ mutants with recombination-related defects in either the exo, β, or γ genes do not grow in ligase-defective hosts. Since the same mutants also fail to grow in polA hosts, this test provides another similarity between lig and polA strains.

d) DNA ligase as an essential function: Because our lig4 mutant grows normally at 42°, despite its very low ligase activity we had severe doubts at one time that ligase is in fact an essential enzyme. Another mutant (ts-7) which has a defective ligase and fails to grow at high temperature had been isolated by C. Pauling. Unfortunately, this mutant was originally found in an E. coli strain in which no genetic analysis is possible; it was thus difficult to tell whether the growth defect was due to the same mutation as the ligase defect.

We have now been able, with some effort, to transduce this mutation into a genetically tractable strain, and to show that all transductants with low ligase activity also fail to grow at 42°. Thus both properties are due to the same mutation, and a certain minimal ligase activity is shown to be required for growth.

e) Physiology: Both lig4 and lig (ts-7) join pulse-labeled DNA fragments into long DNA strands considerably slower than normal strains. Joining is slowed by roughly a factor of ten even under conditions where the growth rate of the strains is normal; thus joining cannot normally be the rate-limiting step in DNA replication.

Our original series of ligase mutants was UV-sensitive, but lig transductants into different genetic backgrounds have lost this property. Evidently our first strains must have contained a second mutation, itself silent, which combined with the lig defect to produce UV-sensitivity.

f) Insertion of ligase gene into phage λ: We have generated λ phages which carry the E. coli ligase gene in place of some non-essential

λ genes. Lysogenization with such a phage suppresses the growth defect of a lig (ts-7) host, demonstrating that the ligase gene is transcribed even in a repressed phage. These phages will both facilitate further genetic studies and provide a rich source for the purification of DNA ligase.

2) Suppressors of gene 32 mutations in T4 (J. W. Little).

The gene 32 product, known to be required for both DNA replication and recombination in T4, is a protein which binds to single-stranded but not double-stranded DNA, and thus makes it possible for DNA to exist in a single-stranded form far below its normal melting temperature (work of B. W. Alberts and colleagues).

The protein may be needed to "prop open" strand-separated regions of DNA so that they can be replicated.

Since this is the only non-enzymatic function known to be involved in replication, it is interesting to study its interplay with the rest of the replication machinery. One approach is to study mutants which, to some extent, spare the requirement for 32-protein. By starting with an amber mutation in gene 32 and an ochre-suppressor host in which a very small (inadequate) amount of 32-protein is made, one can select for phage mutants in other functions which allow phage growth.

The second-site mutants, provisionally termed str, can be separated genetically from the 32 amber locus. They are recessive to str^+, and grow perfectly well; hence str function is not required for phage growth. They do cause a short lag in the onset of DNA synthesis.

The 32 amber str double mutant shows a very long lag in onset of DNA synthesis in the ochre suppressor host. When DNA synthesis finally begins it proceeds at a rate more than double that of the 32 amber alone and the DNA made is packaged into mature phage about as efficiently as wild-type DNA, in contrast to the inefficient packaging of 32 amber DNA. Hence we believe that a qualitative alteration in the DNA of the latter mutant is counteracted by the str mutation. Conceivably the absence of str function causes some imbalance between various processes to be brought back into balance.

Serial No. NIAMD-LMB- 3
1. Laboratory of Molecular Biology
2. Microbial Genetics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Genetics and structure of the oncogenic virus, SV40

Previous Serial Number: NIAMD-LMB-3

Principal Investigator: Robert G. Martin (10/12)

Other Investigators: Dr. Peter McCann (12/12), Dr. Rein Saral (12/12), Dr. Janice Y. Chou (8/12), and Dr. Jesus Avila (2/12)

Collaborators: Dr. William Kidwell (NCI), Dr. Harvey Ozer (Worchester Foundation)

Man Years:

Total:	5-8/12
Professional:	3-8/12
Other:	2

Project Description:

Objectives: To analyze the genetic structure of the oncogenic virus SV40 through the isolation of conditional lethal mutants. Interaction between host functions and viral replication will also be investigated. Structural analyses of the DNA-protein complex of the viral core will be undertaken.

Major Findings: An endonuclease associated with whole SV40 virions has been found and partially characterized (Saral & Martin) in collaboration with Dr. William Kidwell (NCI) and Dr. Harvey Ozer (Worchester Foundation). The enzyme appears to make single stranded nicks, but on prolonged incubation makes more than 1 such nick per SV40 genome. The enzyme is present in virus grown on a number of different host cell lines and is present in all classes of temperature sensitive mutants identified thus far.

A large scale screening procedure for the identification of temperature sensitive mutants (Chou, Avila and Martin) of SV40 has been set up in the attempt to isolate new mutants. A number of possible mutants have been isolated, but further characterization is required.

Characterization of the SV40 DNA core protein complex (McCann and Martin) has been undertaken. Approximately 1% of the SV40 genome is protected from staphylococcal nuclease digestion in the complex. The size of the protected region is approximately 100 nucleotide pairs in length, but it is unclear whether there is 1 such region or 2 such regions per genome. Preliminary

results appear to indicate that the region(s) is a specific portion of the SV40 genome.

Significance to Bio-medical Research and the Program of the Institute:

The significance of this project is that it may lead to some insights into carcinogenesis and other metabolic diseases.

Publications:

Robb, James A., Tegtmeyer, Peter, Martin, Robert G., and Kit, Saul: Proposal for a uniform nomenclature for simian virus 40 mutants. J. Virol. 9: 562-563, 1972.

Robb, James A. and Martin, Robert G.: Genetic analysis of simian virus 40 III. Characterization of a Temperature-sensitive Mutant blocked at an early stage of productive infection in monkey cells. J. Virology (in press).

Serial No. NIAMD-LMB-4
1. Laboratory of Molecular Biology
2. Microbial Genetics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Biochemical control mechanisms in histidine biosynthesis

Previous Serial Number: NIAMD-LMB-4

Principal Investigator: R. G. Martin (1/12)

Other Investigators: Dr. Matthew Rechler (1/12), Dr. C. Bruno Bruni (2/12)

Man Years:

Total:	4/12
Professional:	4/12
Other:	0

Project Description:

Objectives: To characterize the intercistronic region between two genes of the histidine operon of Salmonella typhimurium.

Major Findings: The existence of an intercistronic region between two of the genes of the histidine operon, hisD and hisC, has been established.

Previous results had suggested that a mutant, hisD2352, exhibited reduced expression of the histidine genes distal to hisD because of a frameshift mutation in hisD which allowed translation beyond the normal termination signal and into the intercistronic region. The possibility remained however, that hisD2352 was a deletion mutation extending into the beginning of hisC. It has now been established that the hisC product, IAP: glutamate aminotransferase, has the same 19 amino terminal amino acids in the wild type and hisD2352 strains, eliminating the possibility that hisD2352 is an extended deletion. It has also been demonstrated that translation of the hisD2352 mutation is not required for reduced expression of hisC. It has therefore been proposed that the secondary structure of an intercistronic region is vital for the efficient initiation of translation.

Significance to NIAMD Research: Our understanding of gene function is fundamental to an understanding of genetic disease and prerequisite to genetic manipulation.

References:

Rechler, M.M., Bruni, C.B., Martin, R.G., and Terry, W. An intercistronic region in the Histidine Operon of Salmonella typhimurium. JMB (in press)

Serial No. NIAMD-LMB-5
1. Laboratory of Molecular Biology
2. Microbial Genetics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Correcting the genetic defect in Lesch-Nyhan disease

Previous Serial Number: None

Principal Investigator: Robert G. Martin (1/12)

Other Investigators: Dr. C. Bruno Bruni (3/12), Dr. Janice Y. Chou (4/12)

Man Years:

Total:	8/12
Professional:	8/12
Other:	0

Project Description:

Objectives: To transfer the bacterial genes carrying guanine-hypoxanthine phosphoribosyltransferase to cells from a Lesch-Nyhan patient, deficient in this enzyme.

Major Findings: It has been reported that bacterial genes carrying the galactose operon on a λ phage can be transferred to human cells from a galactosemic patient and yield cells which are now capable of fully metabolizing galactose. Considerable time and effort was spent in trying to reproduce those results. Unfortunately for reasons which remain obscure we have been unable to repeat those results.

However, prior to discovering that the transfer of bacterial genes to known cells could not be repeated, efforts were undertaken to determine where on the bacterial chromosome the gene for guanine-hypoxanthine phosphoribosyltransferase was located. This was a necessary first step to produce a λ phage carrying the phosphoribosyltransferase.

It had previously been reported that S. typhimurium and E. coli strains selected for resistance to 8 azaguanine were not deficient in transferase activity, although mammalian cells selected in the same manner were frequently devoid of this activity. We have demonstrated that S. typhimurium contains two guanine phosphoribosyltransferases. One enzyme is relatively specific for guanine and xanthine transferase activity with low hypoxanthine activity. The other appears to have high hypoxanthine activity, and a mutant (hpt) lacking this latter enzyme has been obtained. It should be relatively simple now to map the hpt site and obtain λ phage carrying this gene.

Since, however, the successful transfer of bacterial genes to mammalian cells can not be repeated, this project has been temporarily dropped.

Significance to NIAMD Research: Had it been possible to transfer bacterial information to mammalian cells it might have been possible to cure the metabolic defect in Lesch-Nyhan syndrome and eliminate this metabolic disease.

Serial No. NIAMD-LMB-6
1. Laboratory of Molecular Biology
2. Microbial Genetics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Genetics and physiology of bacteriophage P1

Previous Serial Number: None

Principal Investigator: J. L. Rosner (12/12)

Collaborators: B. deCrombrugghe (NCI); C. Hidalgo (NIMH); I. Pastan (NCI); W. Shaw (University of Miami).

Man Years:

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives: An understanding of the mechanisms which regulate vegetative growth, plasmid formation, transduction and gene expression by the temperate bacteriophage P1. Special attention has been devoted to:

a) The P1 modification-restriction system.

b) The expression of chloramphenicol transacetylase by P1CM.

Major Findings: a) The bacteriophage P1 modification-restriction system. Bacteriophage P1 directs the production of enzymes which are responsible for the P1-specific modification (methylase) and restriction (endonuclease) system. Studies of the plating behavior of certain P1 mutants on a restriction-proficient but non-immune lysogen reveal that P1 is itself a substrate for the P1 modification and restriction system. Thus, during the course of lytic growth, wild type P1 phage DNA is modified and thereby protected from P1 restriction. Modificationless mutants, however, do not modify their own DNA and so are susceptible to P1 restriction.

Since the P1 modification enzyme converts certain adenine residues in DNA to 6 methyl aminopurine (6MAP), the DNA from wild type P1 might be expected to have more 6MAP than that from modificationless mutants. In experiments carried out with Dr. Cecilia Hidalgo, wild type P1 DNA was found to contain about 540 6MAP, and about 260 5 methyl cytosine (5MC), residues per DNA molecule. However, the amounts of 6MAP and 5MC in the DNA of two modificationless mutants did not differ significantly from that of wild type. It appears

therefore, that the bulk of these methylated bases results from the action of bacterial methylases.

Three types of non-modifying mutants have been identified. P1r^-m^- neither restricts nor modifies but grows and lysogenizes efficiently. P1c2 does not modify nor lysogenize. P1c3 does not modify nor lysogenize and does not grow at low temperature. Yet, on the basis of complementation studies, all three mutant types are defective in the same cistron. Furthermore, pseudo-revertants of c2 and c3 have been isolated and found to be modificationless and restrictionless and now able to grow and lysogenize efficiently. To account for the observations the following model is proposed: Mutations in the modification gene may eliminate the activity of the restriction gene product either wholly (r^-m^-), partially (c2) or not at all (c3 at low temperature). c2 and c3 mutants cannot lysogenize because cell death results from P1 restriction in the absence of P1 modification. c3 mutants do not grow at low temperature because the phage itself is destroyed by an efficient restricting enzyme. Secondary mutations which fully eliminate the restriction function of c2 or c3 mutants eliminate the lethal effects. The possibility that a defective modification enzyme could affect the restriction enzyme activity is suggested by the recent finding that the P1 modification and restriction activities may be isolated in a single multi-enzyme complex. (Brockes et al., Biochemical J.).

b) The expression of chloramphenicol transacetylase by P1CM.

The CM-determinant confers upon bacteria resistance to chloramphenicol by directing the production of chloramphenicol transacetylase (CATase) which inactivates the antibiotic. Harwood and Smith showed that the expression of the CM gene carried by an RTF was subject to catabolite repression. In collaboration with Drs. Pastan, deCrombrugghe and Shaw, the expression of CATase by P1CM is being studied both in vivo and in vitro. The following results have been obtained: 1) P1CM lysogens of mutants deficient in adenylcyclase and/or cyclic AMP binding protein are chloramphenicol-resistant but have lower enzyme levels than wild type lysogens. 2) P1CM DNA in the absence of cAMP directs the production of CATase in the protein synthesizing system described by Zubay. 3) cAMP stimulates a 10-20 fold increase in CATase activity in vitro. Thus, there is a constitutive low-level CATase production which is not sensitive to cAMP control and a high level cAMP-dependent production.

Significance to bio-medical research and the program of the institute:

Restriction and modification is a mechanism whereby cells differentiate between foreign and native DNA. A better understanding of the bacterial systems may facilitate the detection of similar systems in higher organisms. Of particular interest would be the possibility that certain viral diseases are manifestations of restriction-modification systems. The mechanism whereby restriction and modification enzymes recognize particular sequences of DNA is of fundamental importance.

The study of the regulation of CM gene expression may provide insights into the mechanisms of differentiation and of bacterial resistance to antibiotics.

Proposed course of project:

a) Further studies will be undertaken to map the modification and restriction genes on the P1 chromosome. The nature of the modification and restriction enzymes elaborated by the various mutants will be studied in an effort to understand the nature of the multi-enzyme complex.

b) Attempts will be made to isolate promotor mutations of P1CM in which synthesis of CATase is now either completely cAMP independent or completely cAMP dependent. The study of such mutants might lead to understanding of the nature of cAMP control of transcription.

Publications:

Rosner, J. L: The regulation of P1 modification. Abstract Ann. Mtng. Am. Soc. Microbiol. pg. 202: 1972.

Rosner, J. L.: Formation, induction and curing of bacteriophage P1 lysogens. Virology, June 1972 (in press).

Hidalgo, C. and Rosner, J. L.: Methylation of bacteriophage P1 DNA. Virology (in press).

Serial No. NIAMD-LMB-7
1. Laboratory of Molecular Biology
2. Microbial Genetics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Bacterial control mechanisms

Previous Serial Number: None

Principal Investigator: Mark Levinthal (12/12)

Collaborators: Robert F. Goldberger

Man Years:

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives: Pi strains - We are asking the following questions: 1) what is the origin of the pi-his factor? 2) what is the physical nature of the pi-his factor.

The interaction of hisG protein with the DNA of phage $\lambda C_I 857 h80$ susS7 dhis. We are trying to find out the mechanism by which hisG protein intervenes in the control of histidine biosynthesis.

Major Findings: Pi strains - 1. A derivative of hisG203/pi-z which has been cured of pi by ethidium bromide can no longer be induced to produce pi revertants, but still can form other hol^+ revertants.

2. Nitrogen mustard produces pi events efficiently but sulfur mustards do not.

3. Labelling of pi containing strains as well as hisG203 and extraction of DNA followed by alkaline CsCl grsdients shows the presence of circular DNA. Ethidium bromide cured pi strains under the same conditions does not show circular DNA.

G enzyme studies: 1. G enzyme binds with high efficiency to phage DNA carrying his operon.

2. Phage DNA without the his operon will compete away 70% of the binding, while phage DNA carrying the his operon will compete out 100% of the binding.

Proposed course of research:

Pi strains - 1) Analysis of the segregants from integrated pi strains.

2) Further analysis of pi integration and excision in recA strains.

G enzyme - 1) Test binding with phage DNA carrying an O^c mutation in his operon.

2) Test the effect of histidyl tRNA on binding.

Publications:

Levinthal, Mark and Yeh, Jen: The pi-histidine factor of Salmonella typhimurium: A demonstration that pi-histidine factor integrates into the chromosome. J. Bacteriol. 109: 993-1000, 1972.

Serial No. NIAMD-LMB-8
1. Laboratory of Molecular Biology
2. Microbial Genetics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The involvement of bacterial functions in biological processes caused by the infection of bacteriophages

Previous Serial Number: None

Principal Investigator: Toshiya Takano

Man Years:

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives: An understanding of the involvement of bacterial functions in the replication of bacterial viruses. Specifically, attention has been focused on:

a) Molecular genetic studies on the processes of the morphogenesis of the head of bacteriophages.

b) Investigation of a bacterial mutant affecting both plasmid formation and cellular division.

Major Findings: a) Isolation and genetic mapping of bacterial mutants which inhibit the growth of bacteriophages by the block of the morphogenesis of bacteriophage head.

The processes of morphogenesis of bacteriophage head were studied. A bacterial mutant that restricts the growth of phage T4 by the block of head formation, named "mop" (morphogenesis of phage), has been isolated and is located at 83 minutes on the K12 chromosome map. Mutant phages, that can grow on the bacterial mutant were also isolated and the mapping of these phage mutants shows that these mutations are in gene 31 of T4. It is concluded that there is a process in the morphogenesis of phage heads, that is catalysed by a bacterial factor controlled by mop gene, and the defect of the factor is compensated by the mutation of gene 31 of phage T4. This bacterial mutant also restricts phage λ and $\phi 80$, so the bacterial function may be involved generally in the formation of phage heads.

b) Investigation of a bacterial mutant affecting both plasmid formation and cellular division.

Bacteriophage P1 can lysogenize bacteria and replicates as a plasmid synchronous with the cellular division cycle, without any integration into the host chromosome. In a bacterial mutant, lon$^-$, P1 cannot lysogenize and always gets into a vegetative growth cycle (Proc. Nat. Acad. Sci., 68, 1496 [1971]). Recently, a temperature-sensitive lon$^-$ mutant has been isolated. Prophage P1, that was lysogenized at 30°C in the lon ts strain, was induced only by the shift-up of the temperature to 40°C. These results suggest that the product of lon gene acts like a repressor to P1 prophage, and is needed for the maintenance of synchronous replication of P1 prophage.

Significance to bio-medical research and the program of the institute:

Viruses induce a series of new biological processes in the infected host cells. To understand the involvement of host functions in these processes is significantly important, for the future control of these viral processes by bio-medical technology. Specifically, bacteria and their bacterial viruses give simpler and quicker approsches to these goals than mammalian systems, and will provide models for these processes in the mammalian system.

Publications:

Takano, T. and Kakefuda, T.: Involvement of bacterial factor in the morphogenesis of bacteriophage capsid. Nature New Biology (in press).

Takano T.: Bacterial mutants defective in plasmid formation: Requirement for the lon$^+$ allele. Proc. Nat. Acad. Sci. 68: 1469-1473, 1971.

Takano, T.: Current review on lysogenic phages. Protein, Nucleic Acid and Enzymes (Tokyo) 16: 1039-1043, 1971.

Takano, T.: A review on mechanism and regulation of DNA replication. Protein, Nucleic Acid and Enzymes (Tokyo), 16: 1189-1197, 1971.

Serial No. NIAMD-LMB-9
1. Laboratory of Molecular Biology
2. Section on Molecular Genetics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Base Sequence Analysis of R17 Phage RNA

Previous Serial Number: None

Principal Investigator: M. Fuke (8/12)

Other Investigator: J. Tomizawa (1/12)

Man Years:

Total:	9/12
Professional:	9/12
Other:	0

Project Description:

Objectives: Base sequence analysis of R17 phage RNA after development of a method to obtain unique collection of fragments of RNA is aimed.

Methods Employed: Limited and controlled digestion of R17 RNA by ribonuclease T1 and electrophoretic separation of the RNA fragments by acrylamide gel electrophoresis.

Major Findings: A method of fractionation of RNA fragments by gel electrophoresis was established. By limited digestion of R17 RNA at a low temperature which increases enzyme specificity, several large fragments were obtained. Since the bands of the fragments in gel electrophoresis were discrete, each band seems to be a unique collection of molecules. This point is under further investigation.

Significance to Bio-medical Research and the Program of the Institute:

Development of a method which is widely applicable for specific fragmentation of nucleic acids has fundamental importance for nucleic acid chemistry and biology. Sequence analysis of R17 RNA will allow us to explain various functions of the phage based on RNA structure. This research will increase fundamental knowledge of bio-medical knowledge and help to understand RNA oncogenic viruses which carry small single stranded RNA as R17 phage carries.

Proposed Course of Project: This research will be continued. RNA fragments will be identified and base sequence will be analyzed.

Serial No. NIAMD-LMB-10
1. Laboratory of Molecular Biology
2. Section on Molecular Genetics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Replication, Recombination and Repair of Microbial DNA

Previous Serial Number: None

Principal Investigator: J. Tomizawa (11/12)

Other Investigators: Y. Sakakibara (9/12)
Y. Sugino (3/12)
M. Fuke (1/12)

Man Years:

Total:	2
Professional:	2
Other:	0

Project Description:

Objectives: Molecular biological understanding of the mechanisms of replication, recombination and repair of DNA is aimed.

Methods Employed: The mechanisms are studied with E. coli and coli phages. Various genetic tricks are used extensively. Density gradient centrifugation (CsCl and sucrose), differential labelling of DNA with radioisotopes and heavy isotopes were used for isolation of specific type of DNA and its analysis. Structures of DNA were visualized by the electron-microscopic method.

Major Findings: 1) Replication of lambda phage DNA.- Upon infection to bacteria λ phage DNA molecules take a covalently closed circular structure. We have shown that the molecules replicate while keeping a circular structure and segregate into two daughter molecules after completion of one round of replication. To study the mechanisms of initiation of λ DNA replication, λ DNA molecules at the beginning of replication in which newly synthesized progeny strands were labelled with ^{3}H-thymidine were isolated by differential density labelling method we developed. Density gradient analysis of the DNA after heat or alkaline denaturation showed that about 20% of newly synthesized DNA strands were covalently attached to the parental DNA strands. Biological significance of this attachment, especially in connection with initiation of DNA replication, is under investigation.

2) Phage and bacterial mutations which affect replication of lambda phage DNA. -For λ phage replication, gene expressions of λ genome and some

bacterial functions are essential. λ carrying a promoter mutation c17 replicates constitutively and incidentally the infected bacteria are killed. However, when the bacteria are infected with λc17 carrying susO or P which is defective in DNA replication, the cells are not killed. If bacteria which can survive infection of λc17 can be isolated, the cells would have a mutation which affects transcription or expression of functions for replication of phage genomes. Such mutants would provide a way to study the mechanisms of replication of phage genomes. Mutants which survive infection of λc17 and carry the phage as prophage were isolated. The mutants were subjected to further analysis after curing the prophage. A mutant restricts growth of λ, λimm^{434}, λimm^{21} but does support growth of phage 434, 21 and 80. DNA synthesis but not mRNA synthesis is suppressed in the mutant. λ mutants which overcome the restriction by the bacterial mutation were isolated. A λ mutant of this type does not help the growth of sensitive λ in the bacterial mutant. Genetic mapping of the bacterial and phage mutations and analysis of λ phage DNA replication in this mutant are in progress.

3) Electron Microscopic Studies of Replicating and Catenated Colicin Factor E1 DNA Isolated from Minicells. - Replicating and catenated DNA molecules of colicin factor E1 that were isolated from minicells were observed by electron microscopy. Two new replicating structures that have been found include: molecules that contain two untwisted replicated branches of the same length and a twisted unreplicated branch, and a twisted circular molecule with a long tail. Other molecular forms identified include circular dimers, three types of catenated dimers (open-open, open-twisted, and twisted-twisted), catenated dimers in which one of the linked molecules is replicating catenated trimers, and a tetamer. (In collaboration with Dr. J. Inselburg of Dartmouth Medical School.)

4) Studies on recombination-deficient mutants of E. coli. - Recombination-deficient (Rec^-) mutations cause pleiotropic alterations of various properties including those on replication and repair as well as recombination of DNA. Yet the primary function of genes responsible for these alterations have not been elucidated. We isolated temperature-sensitive recA, recB and recC mutants and various properties of bacteria and bacterial extracts were studied. The function of recA gene is still completely unknown but the following results on the functions of recB and recC genes would be conclusive. The ATP-dependent deoxyribonuclease of a temperature-sensitive recB mutant is heat-sensitive, indicating that the recB gene is a structural gene of the enzyme. Although the enzyme of a temperature-sensitive recC mutant is heat-stable, from the low enzyme activity of recBtsrecCts bacteria grown at the permissive temperature, the recC gene is argued to be another structural gene of the enzyme. Another enzymatic reaction must precede the participation of the recBrecC enzyme in the cellular process of repair of u.v. damage. (In collaboration with Dr. H. Ogawa, University of Osaka.

Significance to Bio-medical Research and the Program of the Institute: Understanding of the mechanisms of replication, recombination and repair has prime importance in genetics. Extensive investigation can be made with the system with which we are working. Understanding of these process will have significance for bio-medical research and the program of the Institute.

Proposed Course of Project: Each research project with the exception of (3) will be continued. In vitro studies of these mechanisms may be initiated.

Publications:

Fuke, M., and Inselburg, J.: Electron Microscopic Studies of Replicating and Catenated Colicin Factor El DNA Isolated from Minicells. Proc. Nat. Acad. Sci.USA 69: 89-92, 1972.

Tomizawa, J., and Ogawa, H.: Structural Genes of ATP-dependent Deoxyribonuclease of Escherichia coli. Nature, in press.

Serial No. NIJMD-LMB-11
1. Laboratory of Molecular Biology
2. Section on Molecular Structure
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Crystal structure investigation of antigen antibody interaction.

Previous Serial Number: NIAMD-LMB-7

Principal Investigators: David R. Davies, D. Segal, E. Padlan, E.W. Silverton

Cooperating Units: Dr. Michael Potter, NCI; and Dr. Stuart Rudikoff, NCI.

Man Years:

Total:	3
Professional:	3
Other:	0

Project Description:

Objectives: An investigation of the crystal structure of the Fab fragment of immunoglobulin molecules that bind strongly to small model antigens. In this way it is hoped to map out the binding site of antibodies and to illustrate the mechanism of antigen-antibody interaction.

Methods Employed: Preparation and analysis of pure materials. Crystallization and x-ray diffraction analysis with the heavy atom isomorphous replacement method.

Major Findings: 1) During the past year some seven Fab fragments have been received in reasonably pure form from Drs. Potter and Rudikoff. These are the Fab fragments from mouse myeloma proteins where the intact immunoglobulin is known to bind very strongly to small molecules such as phosphoryl choline. An investigation has then been carried out to determine the most likely method of crystallizing these materials and attempts have been made to produce crystals of a size suitable for x-ray diffraction analysis. To date crystals have been observed for three of these immunoglobulin fragments.

a) MOPC 167 space group P622, cell dimensions a=114, b=114, c=146 Å.
b) McPC 603 space group P63, a=161, b=161, c=60.3 Å.
c) SAPC 10 space group = P6M22, cell dimensions a-139, b=139, c=97.5 Å.

The crystals of McPC603 have been observed to give diffraction maxima at spacings of less than 2.7 Å. We believe that it should be possible to determine the crystal structure to at least a resolution of 3 Å and we have commenced data collection with this end in mind.

In addition, studies have been carried out to show that the crystals bind phosphoryl choline although the binding constant in the crystal is less than that observed in solution (possibly due to the high concentration of ammonium sulfate present in the crystal). A search for suitable heavy atom derivatives for use with the isomorphous replacement technique has commenced and a number of compounds have been found which result in intensity changes.

2) In addition, some work has been continued on the crystal structure of the intact immunoglobulin DOB. Attempts have been made to obtain crystals that diffract better and in addition, a search has been made for other heavy atom derivatives.

Significance to Bio-medical Research and the Program of the Institutes:

An understanding of the molecular basis of interaction between antibody and antigen is fundamental to our understanding of the immune response.

Proposed Course of Project: We shall collect 3-dimensional data for the McPC603 crystals and for suitable heavy atom derivatives with a view to calculating a high resolution electron density map. We also intend to look at other immunoglobulin fragments in order to see whether they can be crystallized.

Publications:

Davies, D. R., Sarma, R., Labaw, L. W., Silverton, E., Segal, D., and Terry, W. D.: X-ray diffraction and electron microscope studies on a crystalline human immunoglobulin. Annals of the New York Academy of Sciences, Vol. 190, 122-129, 1971.

Sarma, V. R., Davies, D. R., Labaw, L. W., Silverton, E. W., and Terry, W. D.: Crystal structure of an immunoglobulin molecule by x-ray diffraction and electron microscopy. Cold Spr. Harb. Quant. Biol., Vol. 36, 413-419, 1971.

Serial Number: NIAMD-LMB-12
1. Laboratory of Molecular Biology
2. Section on Molecular Structure
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: X-ray diffraction Investigation of Proteolytic Enzymes

Previous Serial Number: NIAMD-LMB-8

Principal Investigators: David R. Davies, Gerson Cohen, David Segal, and Ian Swan

Man Years:

Total:	2 1/2
Professional:	2 1/2

Project Description:

Objectives: The determination of the 3-dimensional structure of proetolytic enzymes, in particular, the structure of gamma chymotrypsin and of the acid protease from Rhizopus chinensis.

Methods Employed: 1. The refinement of the coordinates of tosyl γ-chymotrypsin at 2.7 Å resolution has been continued. The binding of specific inhibitors has been investigated by X-ray diffraction with results that are in agreement with our previous work in this field. Attempts have been made to prepare inhibitors that would reveal the position of the leaving group chymotrypsin in catalysis but so far these have not been successful. The kinetics of the hydrolysis of oligopeptides of specific sequence have been extended and have been shown to be completely consistent with the model for substrate binding based on the X-ray diffraction analysis.

2. X-ray diffraction analysis of the crystal structure of the acid protease from Rhizopus chinensis has been continued, and has now reached a stage where we look forward to an electron density map at approximately 3Å resolutions. A low resolution map (6Å resolution) has been calculated but has not been examined in detail because we feel that we shall shortly have a higher resolution map to work with. We have commenced a search for suitable inhibitors of this enzyme with the expectation that a study of the mechanism of this enzyme will be applicable to the mechanism of all of the other enzymes of the general class of acid protease. These include rennin, pepsin and the cathepsins.

Significance to Bio-medical Research and the Program of the Institute: The work on the mode of binding of specific inhibitors to chymotrypsin has turned out to have fairly universal application and has been used to show how trypsin and trypsin inhibitor interact. It has also thrown light on the evolutionary mechanism for the production of enzymes. An understanding of the crystal structure of the acid protease and of its mechanism of action will be of fairly universal interest with regards to the molecular mechanism of action and control of this important class of enzymes.

Proposed Course of Project: When the electron density map of the acid protease from Rhizopus chinensis becomes available, an attempt will be made to interpret this in molecular terms. A search will be made for suitable inhibitors to reveal the location and stereo chemistry of the active site. Experiments will be designed to reveal the mechanism of action of the enzyme.

Publications:

Segal, D. M., Cohen, G. H., Davies, D. R., Powers, J. C., and Wilcox, P. E.: The Stereochemistry of Substrate Binding to Chymotrypsin Aγ. Cold Spring Harbor Symposia on Quantitative Biology. 36: 85-90, 1971.

Segal, D. M., Powers, J. C., Cohen, G. H., Davies, D. R., and Wilcox, P. E.: Substrate Binding Site in Bovine Chymotrypsin Aγ. A Crystallographic Study Using Peptide Chloromethyl Ketones as Site-Specific Inhibitors. Biochemistry. 10: 3728-3737, 1971.

Segal, D. M.: A Kinetic Investigation of the Crystallographically Deduced Binding Subsites of Bovine Chymotrypsin Aγ. Biochemistry. 11: 349-356, 1972.

Swan, I. D. A.: Crystallization and Preliminary Crystallographic data for the Acid Protease from Rhizopus chinensis. Journal of Molecular Biology. 60: 405-407, 1971.

Serial No. NIAMD-LMB-13
1. Molecular Biology
2. Organic Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Chemical and Structural Investigations of Nucleic Acids and Related Substances

Previous Serial Number: NIAMD-LMB-14

Principal Investigator: H. Todd Miles

Other Investigators: Dr. F. B. Howard, Dr. F. Ishikawa, Dr. R. Shoup (LPB), Dr. E. D. Becker (LPB), and Mr. J. Frazier

Man Years
Total: 4 1/2
Professional: 3 1/2
Other: 1

Project Description:

Objectives: Basic chemical, biochemical, and structural studies of nucleic acids and related substances.

Methods: Infrared spectroscopy, nuclear magnetic resonance, spectroscopy, chemical synthesis and structure elucidation, optical rotation, ultraviolet spectroscopy, use of enzymes for synthesis and structure study.

Major Findings: Two stranded polynucleotide helices may, in principle, exist in structurally isomeric forms having different hydrogen bonding arrangements and different strand polarities. Available evidence has indicated, however, that previously known 1:1 complexes are based solely upon a Watson-Crick bonding scheme. It has not been known whether structures other than this most stable one were fundamentally incapable of existence or whether they might be separated from the commonly obserbed one by relatively small differences of energy. We have recently demonstrated for the first time the formation of two-stranded A·U polynucleotide helices having non-Watson-Crick hydrogen bonding. There is therefore no steric or energetic prohibition of non-Watson-Crick structures in two stranded helices. It appears that the energy difference between the two helical structures in systems which could, in principle, exist in either form may not be large. It is probably the cooperativeness of the transition rather than the magnitude of the energetic difference which is responsible for exclusive formation of a single structure rather than a mixture of two forms. Steric control of the hydrogen bonding site in the present study was achieved by

introducing a dimethylamino substituent into the 2-position of poly A, thus blocking hydrogen bonding to the N_1 position of the purine residues. Placing a substituent at N_1 would have blocked hydrogen bonding at that position but would also have introduced a positive change on the purine ring, thus preventing a relevant interaction from occurring.

In studies of the interaction of poly rT with poly rA we have found that there is initial rapid formation of both 2 and 3-stranded material at the 1:1 ratio in 0.1 M Na^+. The 3-stranded complex is metastable with respect to the 2-stranded under these conditions, but the only available path to the equilibrium species is a very slow (3 or more days) rate-limiting displacement reaction: rA·2rT+rA→ 2 rA·rT. Preliminary experiments suggest that this phenomenon of rapid formation of a metastable 2:1 complex is probably general in systems which are capable of forming both 1:1 and 2:1 complexes. A practical consequence of this kinetic behavior is that most of the presumed equilibrium mixing curves which have been reported with such systems were not carried out under equilibrium conditions. Some of the erroneous results in the literature are very probably due to this kinetic artefact.

The amino groups of purine and pyrimidine bases are presumed to undergo relatively free rotation when they are not paired in helical complexes. We have recently observed high field proton NMR spectra, however, demonstrating that rotation of the amino group in cytosine is in fact restricted. Since the frequencies of the two amino protons are nearly equal, and the use of ^{15}N labelling of the cytosine and a 220 M hz NMR spectrometer were necessary to show clear resolution of the proton signals.

We have also found restricted rotation in monomethylaminocytosines, in contrast to previous reports, and have shown that the predominant geometrical isomer (95%) has the methyl group <u>syn</u> to N_3 (the position at which base pair formation occurs).

A related study was carried out on restricted rotation of dimethyl-aminocytosine derivatives. Though these molecules are incapable of base pairing they are suitable for quantitative determinations or rotational barriers, unlike the unsubstituted amimes. From these NMR studies of model systems we have reached a conclusion of importance for polynucleotide interactions. Other workers had reported that poly C substituted with a single methyl on the amino group did not interact with poly I, though, in principle, it should be capable of doing so. It appears that the reason for this failure is the predominance of the geometrical isomer incapable of bonding and the short lifetime ($\sim 10^{-3}$ sec) of the conformation which would be capable of hydrogen bonding.

Publications:

Howard, F. B., Frazier, J., and Miles, H.T.: J. Biol. Chem. 246: 7073-7086, 1971.

Shoup, R. R., Becker, E. D., and Miles, H. T.: Biochem. and Biophys. Res. Comm. 43: 1350-1353, 1971.

Shoup, R. R., Miles, H. T., and Becker, E.D.: J. Phys. Chem. 76: 64-70, 1972.

Ishikawa, F., Frazier, J., Howard, F. B., & Miles: J. Mol. Biol., in press.

Ross, P. D., Scruggs, R. L., Howard, F. B., and Miles, H. T: J. Mol. Biol 61: 727-733, 1971.

Serial No. NIAMD-LMB-14
1. Laboratory of Molecular Biology
2. Physical Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Calorimetric Studies of Human Blood Platelets

Previous Serial Number: NIAMD:LMB:None

Principal Investigator: Philip D. Ross

Other Investigator: A. P. Fletcher

Cooperating Unit: The American National Red Cross Blood Research Laboratory

Man Years

Total: 1
Professional: 1

Project Description:

Objectives: To understand the changes in metabolism of human blood platelets in the presence of agents known to induce and inhibit platelet aggregation.

Methods Employed: The heat production of human blood platelets was observed calorimetrically. Suspensions of blood platelets were mixed with agents known to induce or inhibit platelet aggregation and the resultant thermokinetic behavior was observed.

Major Findings: Simple agitation of platelets, in the absence of any additive, causes the production of heat. Superimposed upon this basal level each inducer of platelet aggregation (Cd^{++}, Mn^{++}, ADP and collagen) exhibits a uniquely characteristic thermal behavior. The aggregation caused by the action of thrombin produces a large increase in the thermal activity of platelet suspensions which is strongly Ca^{++} dependent. It appears that most of the increased heat production in the presence of thrombin arises from increased metabolic activity of platelets rather than from aggregation per se. The action of thrombin upon platelets in the presence of specific inhibitors of glycolysis and oxidative phosphorylation exhibited striking and characteristic thermokinetic behavior associated with the energetic and time sequence of those pathways to the overall metabolism.

Significance to Bio-medical Research and the Program of the Institute: The methods developed in this study are directly adaptable to the investigation of substances of potential therapeutic value and may help

in elucidating the sequence of events involved in hemostasis and thrombogenesis.

Proposed Course of Project: This work is being continued. Two papers are being prepared for publication.

Serial No. NIAMD-LMB-15
1. Laboratory of Molecular Biology
2. Physical Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Thermodynamic Effects of Exposing Nucleic Acid Bases to Water: Solubility Measurements in Water and Organic Solvents.

Previous Serial Number: NIAMD-LMB-None

Principal Investigators: Robert L. Scruggs, Eugene K. Achter and Philip D. Ross

Man Years

Total: 2
Professional: 2

Project Description:

Objectives: To delineate, in thermodynamic terms, the effect of exposing nucleic acid bases to water.

Methods Employed: The equilibrium solubilities of adenine, cytosine, and uracil in water and organic solvents have been determined as a function of temperature.

Major Findings: Transfer of a nucleic acid base from an organic environment into water is characterized by positive values for ΔH and ΔS. It is concluded from this result that the overall interaction between nucleic acid bases and water cannot be hydrophobic. If the observed effect represents structure breaking in water by nucleic acid base, this process would account for a major portion of the large, positive melting entropy of DNA, and would also contribute substantially to the melting enthalpy.

Significance to Bio-medical Research and the Program of the Institute: These measurements provide fundamental thermodynamic information on a model system relevant to the denaturation of organized polynucleotide structures.

Proposed Course of Project: This work is completed and has been submitted for publication.

Serial No. NIAMD-LMB-16
1. Laboratory of Molecular Biology
2. Physical Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Aggregation of Polynucleotides at Acid pH.

Previous Serial Number: NIAMD-LMB-12

Principal Investigator: Steven B. Zimmerman

Other Investigator: None

Man Years

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives: To describe and better understand the mechanisms involved in the aggregation of polynucleotides at acid pH.

Methods Employed: Standard techniques of biochemistry and biophysics.

Major Findings: Homopolyribonucleotides (poly A, poly G, poly C and poly U) precipitate at well defined specific acidic pH values. Random copolymers containing equal amounts of two bases (e.g. poly AG, poly CU, etc.) precipitate at pH values intermediate between those of the pertinent homopolymers and the exact pH of precipitation is a function only of the base with the lower pK; these results are consistent with precipitation being dependent upon groups which titrate independently of each other and at the same pH values whether in a homopolymer or a heteropolymer. This suggests use of fractional acid precipitation to separate species with differing average base compositions; however, a model system such as equal amounts of poly A plus poly G precipitates as a whole at the same pH as does poly AG. Hence at least for some bases the neutralization of the bases occurs independently of the actual backbone to which the bases are attached, and precipitation occurs when the overall charge neutralization of the solution reaches a given level.

The acid precipitates of a number of polyribonucleotides have been examined as powders by X-ray photography. There is no evidence for a generally occurring secondary structure; however, several polynucleotides appear to retain in the precipitate the structure which they have in solution at acid pH.

Freezing acidic solutions of a wide variety of polynucleotides causes precipitation under conditions where the polynucleotides are soluble in the absence of freezing. These precipitates have considerable gross morphological regularity; e.g.; poly A was quantitatively converted into thin flat "plates" of irregular outline. X-ray techniques were used to demonstrate the existence in these "plates" of the double-stranded acid poly A structure delineated from fiber diffraction patterns of acidic poly A by Rich, Davies, Crick and Watson. The "plates" have partial molecular orientation: the fiber axis of the poly A tends to lie in the plane of the plate but to be oriented at random in that plane.

Proposed Course of Project: The patterns of precipitation of model polyribonucleotides will be further determined to test the generality of precipitation as a whole rather than selective pH precipitation. The theory of coprecipitation and coacervation of Overbeek and Voorn will be applied to rationalize this behavior. Fractionation of soluble species in the pH range where partial titration has occurred will be attempted. Fractionation by molecular weight at a given pH probably occurs and whether this can be useful in practice will be determined.

Serial No. NIAMD-LMB-17
1. Laboratory of Molecular Biology
2. Section on Physical Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Structure of Single Stand Polynucleotides in Solution

Previous Serial Number: NIAMD-LMB-9

Principal Investigator: Gary Felsenfeld

Other Investigators: E. Achter
B. J. Stannard

Man Years

Total:	2.5
Professional:	1.5
Other:	1

Project Description:

Objectives: To elucidate the structure of single strand polyribonucleotides in solution.

Methods Employed: Theoretical analysis, light scattering, viscometry, spectrophotometry, sedimentation velocity, sedimentation equilibrium.

Major Findings: We have continued our studies of polyadenylic acid (poly A) as a model of single strand polynucleotides. In order to examine the role of solvent upon structure, we have measured the unperturbed dimensions of poly A under ideal solvent conditions in aqueous solutions of lithium perchlorate. We find that the polymer dimensions do not differ significantly from those obtained for poly A in sodium chloride when compared under conditions involving the same degree of ordering (stacking). We conclude that the nature of the counterion is not significant to the conformation of the poly A backbone.

We have also begun work on the conformation of poly A in the non-aqueous solvent, formamide. Our preliminary results suggest that even this radical change in the solvent environment has very little effect upon the rigidity of the backbone, which our earlier studies had shown was a fundamental property of single strand polynucleotides in aqueous solution.

Significance to Bio-medical Research and the Program of the Institute: The structure of polynucleotides in solution is directly related to function. In transfer RNA, for example, the folding of the molecule appears to be

related to its biological function, while the ability of DNA to act as a template for DNA replication or messenger RNA synthesis depends upon the ability of DNA to undergo structural changes. Our finding that polyribonucleotides are (quite apart from the rigidity provided by base stacking) extraordinarily rigid molecules is quite important in understanding the rules that govern polynucloetide structure formation. Thus, theories concerning the processes of strand separation and combination in multistrand structures such as DNA must now take this property into account, since it has a profound influence upon these processes.

Proposed Course of Project: Studies of polynucleotide dimensions in formamide will be continued. Studies of single strand polydeoxyribonucleotides will be carried out to examine the role of the ribose group in stabilizing the polynucleotide backbone.

Serial No. NIAMD-LMB-18
1. Laboratory of Molecular Biology
2. Section on Physical Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: DNA-Histone Interaction and Chromatin Structure

Previous Serial Number: NIAMD-LMB-10

Principal Investigator: Gary Felsenfeld

Other Investigators: Robert J. Clark
Howard Cedar

Man Years:

Total: 2 1/2
Professional: 2 1/2

Project Description:

Objectives: The objective of this project is to study the interactions of histones with DNA, in order to determine the structure of chromatin and the role of protein-DNA interaction in the regulation of DNA activity in the nucleus.

Methods Employed: Spectrophotometry, analytical ultracentrifugation, dialysis equilibrium, and other standard techniques are used to study the binding of charged molecules to DNA, and the composition of the regions of DNA to which these molecules are bound. Standard chromatographic and electrophoretic techniques are used in purification and identification of histones. RNA polymerase is purified by classical enzymological techniques and its activity examined by membrane filtration, sucrose gradient sedimentation, or radioisotope incorporation.

Major Findings: We have developed a method for removing selectively from calf thymus chromatin the lysine-rich and slightly lysine-rich histones without disturbing the arginine-rich histone fractions (Histones III and IV). As we showed earlier, the usual methods for selective histone removal result in rearrangement of those histones remaining bound to DNA. We are able to show that using the methods we have developed, the arginine-rich histones do not undergo exchange, and are bound to their original sites on DNA. By digestion with the enzyme staphylococcal nuclease, we are then able to remove all but the DNA protected by histones III and IV. We find that the protected DNA, and hence the binding sites of these histones, is unusually rich in guanine-cytosine (G·C) base pairs.

We have also studied the binding of E. coli RNA polymerase to chromatin, and compared it to the binding of this enzyme to protein-free DNA. We have employed a number of titration methods never before used on chromatin. We find that chromatin from various sources binds about an order of magnitude less polymerase per nucleotide than the comparable DNA. We have chosen our conditions so that there is no opportunity for binding of several enzyme molecules to the same site, a shortcoming of previous studies of this sort by other workers. We conclude that chromatin proteins prevent binding of polymerase, rather than merely preventing RNA chain propagation as had been supposed previously.

Significance to Bio-medical Research and the Program of the Institute:

Chromatin is the DNA-protein complex isolated from the nuclei and eukaryotic organisms. On the basis of chromatin's reduced priming activity in vitro as a template for DNA-dependent RNA polymerase, it has been suggested by many investigators that the proteins of chromatin are responsible for the selective reduction in genetic activity that gives rise to cellular differentiation. The mechanism of cellular differentiation is one of the major problems of modern biology. The methods we have developed make it possible to isolate the various protein fractions attached to their original binding sites in chromatin. It is our hope that this will permit elucidation of the role of chromatin proteins in differentiation. The identification of G·C rich DNA as the binding site of arginine-rich histones is the first identification of an architectural detail of chromatin. It remains to be shown whether this particular detail is related to chromatin structure or to regulation of transcription (or both). The use of the polymerase binding assay we have developed should help to answer this question.

Proposed Course of Project: We will study the activity of the so-called 'active' and 'inactive' fractions of chromatin by the methods we have developed. Chromatin from more highly differentiated tissue (such as avian erythrocytes) will be studied, and the products of transcription in vitro of various derivatives of chromatin compared with the in vivo messenger RNA.

Serial No. NIAMD-LMB-19
1. Laboratory of Molecular Biology
2. Section on Theoretical Mol Biol.
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Ion transport across the nerve axon membrane

Previous Serial Number: None

Principal Investigator: Dr. T. L. Hill (8/12)

Other Investigators: Dr. Y. Chen (8/12), Dr. E. Paul (12/12), Dr. R. Blumenthal (8/12)

Cooperating Units: Lab of Biophysics, NINDS (Dr. Blumenthal has collaborated in the experimental work of Drs. Ehrenstein and Lecar). Serial No. NDS-(I)-65 LB/OC 1240

Man Years:

Total:	3
Professional:	3
Others:	0

Project Description:

Objectives: To attempt to interpret experimental kinetic, steady-state, and noise data on K^+ (especially) and Na^+ transport across the nerve axon membrane in terms of a model in which the channels are protein complexes undergoing voltage-dependent conformational changes.

Major Findings: The noise power spectrum from the open-close Hodgkin-Huxley kinetics of K^+ channels has been derived. The result does not correspond to the experimental 1/f spectrum. Hence the latter presumably originates in ion transport through channels which are already open. (Hill & Chen).

Two theoretical models have been studied which might account for the observed delay in the appearance of a K^+ current when depolarization to around the Na^+ potential is preceded by a considerable hyperpolarization. (Hill & Chen)

The free energy (ΔG) and the activation free energies for the subunit conformational changes, presumably responsible for the opening of K^+ and Na^+ channels, can be deduced from experimental data of the Hodgkin-Huxley type. These free energies are functions of V (membrane potential). In particular, ΔG is a simple quadratic function of V. Most likely, the linear term is due to a Δ(net charge) or Δ(dipole moment) effect and the quadratic term to a Δ(polarizability) effect. (Hill & Chen)

As an off-shoot of the nerve membrane work, we have studied the exact kinetics (via computer) of small cooperative Ising systems. We have investigated particularly the departure of such systems from internal equilibrium, as a function of time. (Paul & Hill)

Collaborative experimental work (with Ehrenstein and Lecar in NINDS) has been carried out on the biophysics of artifical black lipid membranes "decorated" with EIM (a protein which apparently forms channels for ion transport). (Blumenthal)

Significance to Biomedical Research and the Program of the Institute:

An understanding of the operation, at the molecular level, of a normal nerve membrane is prerequisite to a rational attack on various neurological and neuromuscular abnormalities.

Publications:

Hill, Terrell L. and Chen, Yi-der: On the theory of ion transport across the nerve membrane. IV. Potassium ion kinetics and noise. Biophysical J. (in press).

Hill, Terrell L. and Chen, Yi-der: The theory of ion transport across the nerve membrane. V. Two models for the Cole-Moore K^+ hyperpolarization delay. Biophysical J. (in press).

Hill, Terrell L. and Chen, Yi-der: On the theory of ion transport across the nerve membrane, VI. Free energy and activation free energy of conformational change. Proc. Nat. Acad. Sci. (in press).

Paul, Edward and Hill, Terrell L.: The kinetics of small ising systems, I. Deviations from internal equilibrium in a tetrahedral model. Proc. Nat. Acad. Sci. (in press).

Serial No. NIAMD-LMB-20
1. Laboratory of Molecular Biology
2. Section on Theoretical Mol. Biol.
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Statistical thermodynamics of polynucleotide - complementary monomer interactions

Previous Serial Number: None

Principal Investigator: Dr. T. L. Hill (4/12)

Other Investigators: Dr. Y. Chen (4/12)

Man Years:

Total:	2/3
Professional:	2/3
Others:	0

Project Description:

Objectives: (a) To clarify the thermodynamic procedures which should be used in comparing optical, calorimetric, and binding measurements on these systems; and (b) to attempt to interpret these experiments in terms of a statistical mechanical model.

Major Findings: The necessary statistical mechanical methodology has been worked out. Computations based on a particular model can now be initiated. The thermodynamic part of the project is well under way. Preliminary results have already led to a considerable clarification of the inter-relations between optical, calorimetric, and binding data.

Significance to Biomedical Research:
This project will contribute, through interpretation of experimental data, to our understanding of the forces involved in stabilizing polynucleotide and nucleic acid molecules in solution.

Publications:

Hill, Terrell L.: Simplified method in polynucleotide helix-coil transition theory including binding of complementary monomer. Proc. Nat. Acad. Sci. (in press).

Annual Report of the

Mathematical Research Branch

National Institute of Arthritis and Metabolic Diseases

Kinetic studies of the conversion of prothrombin to thrombin in an activated factor X system have been carried out. Experiments designed to test superposition and linearity in the initial conditions show the system to be linear when the initial conditions are suitably restricted. The final value and the time course of thrombic activity in the (initial) presence and absence of soybean trypsin inhibitor are consistent with the formation of at least one intermediate which is irreversibly bound by inhibitor thereby decreasing both rate of formation and final yield of thrombin. The greatly increased degree of inhibition observed when inhibitor is added during the course of conversion requires the hypothesis that at least one of the intermediates possesses thrombin activity. (Dr. W. London, Dr. N. R. Shulman, CH, and Dr. J. Hearon)

Studies of mathematical models for recurrent epidemics have continued. In particular the time dependence of the coefficient of infectivity (β) has been analyzed. It can be shown (analytically for differential equation models, by simulation for the differential-delay model) that recurrent epidemics similar to those observed in measles, mumps and chicken pox are predicted only if β is low in the summer months and high for the rest of the year. Fits to actual data show β to drop about 50% during the summer months. It is suggested that sociologic patterns of susceptibles and infectives explain this drop. (Dr. W. London and Dr. J. Yorke, University of Maryland)

A computer simulation of the shape change of the neural plate of the newt has been greatly improved. The simulation presupposes minimal physical mechanisms for embryogenesis and is designed to test the adequacy of such mechanisms to describe early morphogenesis of the brain and cord. (Dr. R. Gordon, Guest Scientist)

Studies of the resolution of Algebraic Reconstruction Technique (J. Theor. Biol. 29, 471, 1971) include improvement of the computer program SNARK. The program is to be used for three-dimensional reconstruction of scintillation data from tumor patients and for implementation of a theory of visual processing. The theory postulates that the visual cortex reconstructs the retinal image in a manner basically similar to ART. (Dr. R. Gordon, Guest Scientist)

Work has continued on the mathematical theory of renal function. The initial phase of the analytical investigation of the "central core" model of the medullary concentrating system has been completed and is being written up for publication. In this model descending Henle's limbs (DHL), ascending Henle's limbs (AHL), and collecting ducts (CD) exchange with a central vascular core (VC) formed by vasa recta loops---assumed so highly permeable that the core functions as a single tube open at the cortical end, closed at the papillary. Solute supplied to the VC primarily by AHL increases VC osmolality and so extracts water from DHL and CD, increasing their osmolality while diluting AHL fluid. This single effect multiplied by the counterflow

arrangement leads to a high papillary osmolality in all structures. Some of the solute may enter DHL to be recycled. For all degrees of recycling the osmolality ratio r of final urine to plasma is given by the dimensionless mass balance equation

$r = 1/[1 - (1 - f_W)(1 - f_U) f_T]$, where f_T is the fractional net solute transport out of AHL, $f_U/(1 - f_U)$ is the ratio of urine flow to AHL flow at the papilla, and f_W is the fraction of AHL transport wasted by vascular washout. Solution of the differential equations describing the system permits calculation of f_W, f_U, and f_T from membrane parameters and boundary conditions. In single solute systems energy requirements for transport out of AHL decrease from outer to inner medulla. In two solute (e.g., salt and urea) systems mixing in the central core can supply part of the energy for the final concentration of urine. Urea cycling, regulared by ADH, allows active Na^+ transport in the outer medulla and cortex to be used for concentration in the inner medulla. (Dr. J. Stephenson, NHLI, Associate Member, MRB)

A paper on the diffusion and consumption of oxygen in regular polygonal regions has been published (Math. Biosci. 13, 55, 1972) detailing among other factors, the influence of relative rates of axial and radial diffusion. (Dr. J. M. Gonzalez-Fernandez and Mrs. S. Atta)

The dynamics of substrate transport in heterogeneous capillary networks has been analyzed. The expression obtained last year for extraction from an arbitrary directed network has been used to determine conditions characterizing optimum (maximum) extraction for substrates obeying linear kinetics. This work has been written up for publication and further work has extended these results in part to substrates obeying complex nonlinear kinetics. (Dr. J. M. Gonzelez-Fernandez and Mrs. S. Atta)

The large linear systems which occur in the numerical solution of partial differential equations (in particular those describing the diffusion-reaction problem at the capillary level) are generally solved by an iterative relaxation method. A new algorithm has incorporated into the iterative method which optimizes the relaxation factor thus giving the best rate of convergence of the iterates to the solution. (Dr. J. M. Gonzalez-Fernandez and Mrs. S. Atta)

The boundary value problem for exponentially branched and tapered neurons was formulated in 1962. Previous emphasis has been upon that class of dendritic trees for which an equivalent cylinder exists. Analytic solutions and computed numerical examples have been obtained for exponential cases which infringe the equivalent cylinder condition. Besides the inherent interest in these results, they allow assessment of the error resulting from the equivalent cylinder assumption which underlies a now widely used method

for estimating time constants and effective length of dendritic trees. (Dr. W. Rall and Dr. S. Goldstein)

A mathematical model for nerve action potential was previously reported (Rall, 1965) which is simpler than the Hodgkin-Huxley model but which correctly reproduces the time sequence of voltage and conductance charges. A stability analysis of the singularities of the descriptive autonomous differential equation system has been carried out and has led to new theoretical insights into this general class of systems as well as specific information for the use of the model. (Dr. W. Rall and Dr. S. Goldstein)

The incorporation of tagged leucine into very low density (I) and low density (II) lipoproteins has been studied. I is apparently transformed to II via two pathways, one a direct removal of most tryglyceride and the other a stepwise removal. Three isolable moieties of II (S_f-17, 10 and 4) follow a direct product precursor relation. (Mr. R. Phair, Dr. W. Fisher, Visiting Scientist, LCB, and Dr. M. Berman)

An insulin subsystem resolvable into three compartments has been defined. The rate of utilization of glucose correlates most closely with the "slowest" of the three compartments. (Dr. M. Berman with Dr. R. Sherwin, NICHD)

Lithium effects on iodine kinetics have been further studied. In addition to the effect (reported last year) on the hydrolysis of thyroglobulin, a previously unsuspected effect on thyroid hormone degradation has been found and confirmed by more direct experiment. (Dr. M. Berman, Dr. R. Temple, J. Wolff and J. Robbins, CE)

It has been shown that if the matrix A of the system $\dot{x}=Ax$ is Hessenberg with $a_{i,j} \geq 0$ for $i \neq j$, then when $x(0) = [1,0,\ldots,0]$ an explicit representation can be written for the last component of x. This representation underlies an extensive analysis of path lengths and initial derivatives in compartmental systems (Math. Biosci. 13, April, 1972) and a general formulation of the distribution of residence times in compartmental systems (Math. Biosci. 13, April, 1972). (Dr. J. Hearon)

It has been shown that as a consequence of the Frobenius theorem on nonnegative matrices a matrix with nonnegative off diagonal entries is permutationally similar to a triangular matrix if it has a single root. As a result, many apparently disparite graph-theoretic results can be shown to follow basically from the classical theorem (Compartmental Matrices with Single Root and Nonnegative Nilpotent Matrices: Math. Biosci. 13, 1972, to appear). (Dr. J. Hearon)

Serial No. NIAMD, ODIR, MRB-1
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Mathematics of kinetics and reaction-transport systems

Previous Serial Number: SAME

Principal Investigator: John Z. Hearon

Man Years:

Total:	1.3
Professional:	1
Others:	.3

Project Description:

Objectives: The objectives of this research remain substantially as stated in previous reports.

Major Findings: In addition to consultative and collaborative activity, efforts during the past year were concerned with the following work:

Linear Hessenberg Systems.

It has been shown that if in the system $\dot{x} = Ax$ the matrix is lower Hessenberg and the initial vector is $[1,0,\ldots,0]$ then the last component of x can be written $x_n(t) = \alpha e_1 * e_2 * \ldots * e_n$, where α is a constant and $e_i = \exp(\lambda_i t)$ with the λ_i the roots of A. A large number of theoretical and practical consequences of this have been reported in a forthcoming paper. Of particular interest and reported in a separate paper is the fact that the above representation allows the distribution of residence times (and all moments of the distribution) in a large class of compartmental systems to be formulated in a simple manner.

Nonnegative Nilpotent Matrices.

In connection with a study of the possible number of precursors of a substance (W. London, annual report, 1970) the question arose as to the circumstances under which a compartmental matrix could have a single n-fold root. Surprisingly, this question has been settled on the basis of the Frobenius theorem on nonnegative matrices. Further, it then follows that a variety of interrelated graph-theoretic and matrix theorems result from the classical theorem. These results are summarized in a forthcoming paper.

Minimum Equation and Directed Graph.

A well known and important theorem states that if A is nonnegative and irreducible and has minimum polynomial of degree m, then for each (i,j) there is a q such that A^q has a strictly positive entry in the (i,j) position and $q \leq m-1$. A restatement is that in the directed graph G(A) of A, no distance between vertices can exceed m-1. It has now been shown that for an arbitrary complex matrix if in G(A) i is connected to j and cofactor $a_{ij} \neq 0$ then the distance i to j is not greater than m-1. This theorem includes above stated result as a special case.

Invariant Subspaces.

In the study of lumping in linear systems reported last year an important result of some considerable practical "diagnostic" utility was that: M lumps A if and only if x(0) in null space A implies x(t) in null space A where $\dot{x}(t) = Ax(t)$. The sufficiency proof which was rather complex can be based on the following theorem which is of more general interest in the study of linear systems.

Consider $\dot{x} = Ax$, $x(0) = x^0$, and a given subspace $\mathcal{S}$. Then $x^0 \in \mathcal{S}$ implies $x(t) \in \mathcal{S}$ if and only if $\mathcal{S}$ is an invariant subspace under A. In particular the range of A and any subspace spanned by a subset of eigenvectors of A are invariant subspaces. The importance of the last special case has long been recognized (N. Y. Acad. Sci. 108, 36, 1963) in linear analysis.

Publications:

Hearon, J. Z.: Compartmental matrices with single root and nonnegative nilpotent matrices. Math. Biosci. (In Press).

Hearon, J. Z.: Residence times in compartmental systems and the moments of a certain distribution. Math. Biosci. (In Press).

Hearon, J. Z. and London, W. P.: Path lengths and initial derivatives in arbitrary and Hessenberg compartmental systems. Math. Biosci. (In Press).

London, W. P. and Hearon, J. Z.: Estimation of the number of precursors in a sequence of first order reactions. Math. Biosci. (In Press).

Serial No. NIAMD, ODIR, MRB-2
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Mathematical formulation and analysis of problems relevant to experimental neurophysiology

Previous Serial Number: SAME

Principal Investigator: Wilfrid Rall

Other Investigators: Steven Goldstein

Man Years:

Total:	2.3
Professional:	2.0
Others:	.3

Project Description:

Over the past several years, this project has been concerned with constructing, developing and testing a group of interrelated mathematical models. This group of models provides a theory that can account for many different sequences of events in a dendritic neuron, and in certain populations of such neurons. Computational experiments performed with these models provide theoretical predictions that have been compared with the results of neurophysiological experiments and neuroanatomical observations. Also, insights have been obtained, which have led to new hypotheses, new interpretations, and to the design of new experiments.

1. Implications of exponential taper and branching in neurons (with Steven Goldstein). Although the boundary value problem for exponential taper and branching was formulated in 1962, previous emphasis has been upon the class of dendritic trees which can be transformed to equivalent cylinders. Now we have obtained the solutions and computed numerical examples for several cases of departure from the equivalent cylinder constraints. These solutions are of considerable interest in themselves, but of most importance to us is the estimation of errors that arise when the simpler equivalent cylinder theoretical results are used to analyze experimental data. Such analysis has now been carried out in several laboratories, here and abroad, to estimate membrane time constants and the effective length of dendritic trees. Fortunately, the rigorous solutions for taper show that the deviation from equivalent cylinder results is small in most

Serial No. NIAMD, ODIR, MRB-3
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Mathematical description of the transport-chemical reaction kinetics of substances in the blood-capillary-tissue complex

Previous Serial Number: SAME

Principal Investigator: Jose M. Gonzalez-Fernandez

Other Investigators: Susie E. Atta

Man Years:

Total:	2.3
Professional:	2
Others:	.3

Project Description:

Objectives: The broad objectives of this research remain substantially the same as the ones stated in the previous reports.

Major Findings:

Steady state:

1. A paper containing the results on the diffusion consumption of oxygen in regular polygons was published (1).

2. The dynamics of substrate transport in heterogeneous capillary networks was analyzed. A paper containing the results characterizing the maximal transport for substrates of simple kinetic behavior is written and it is to be submitted for publication.

Work in progress has partly extended the above results to the transport of substrates of complex kinetic behavior.

Nonsteady state:

To the general formulation for the nonsteady state extraction of oxygen as discussed in the previous report, the influence of the myoglobin and oxymyoglobin kinetics and diffusion have been incorporated.

Numerical Analysis:

Large linear systems of equations, such as those obtained directly from the numerical solution of partial differential equations are in general solved by a relaxation iterative method. A new algorithm incorporated into the iterative method is being developed for optimizing the relaxation factor that gives the best rate of convergence to the solution of the system.

Publications:

(1) Gonzalez-Fernandez, J. M. and Atta, S. E.: Concentration of oxygen around capillaries in polygonal regions of supply. Math. Biosci. 13: 55-69, 1972.

Serial No. NIAMD, ODIR, MRB 4.1
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Analysis of kinetic data and modeling

Previous Serial Number: SAME

Principal Investigator: Mones Berman

Other Investigators: R. Phair (NIAMD); R. Sherwin, P. Insel, R. Andres (NICHD); R. Levy, D. Bilheimer (NHLI); W. Fisher (Univ. of Florida)

Man Years:

Total:	1.4
Professional:	1.4
Others:	0

Project Description:

Objectives: Analysis of data on metabolic systems, and the further development of modeling techniques through the use of the SAAM computer program. Major emphasis went into the lipoprotein system and the glucose-insulin control system.

Major Findings: The incorporation and metabolism of leucine as a tag of VLDL and LDL proteins has been investigated (R. Phair, W. Fisher). The major findings are: 1) VLDL seems to undergo a transformation to LDL via 2 pathways: one a direct splitting off of most of its triglycerides (TG) and transformation into a LDL (S_f4) molecule and the second a gradual loss to TG leading eventually to LDL (S_f17) moiety. 2) The three LDL moieties isolated (S_f17, S_f10, S_f4) follow a direct precursor - product relationship suggesting a gradual loss of TG. 3) The protein kinetics of VLDL and LDL seem consistent as the

previously studied TGFA kinetics, lending support to the above mechanisms.

More extensive studies of VLDL label (Phair, Bilheimer, Levy) including its D and B apo-proteins are still in progress but no major conclusions have been drawn.

Analysis of kinetic data and modeling:

The insulin subsystem was defined (Sherwin) and the major findings are 1) the metabolism of insulin is linear (proportional to concentration); 2) the subsystem is resolvable into 3 compartments; 3) the rate of glucose utilization correlates best with the slowest of the three insulin compartments.

The glucose subsystem is presently being defined and the sites of action of insulin (coupled with above findings for insulin) in the glucose subsystem are being investigated (Insel).

In collaboration with Dr. A. Dubois, a visiting scientist from Belgium, a model is being developed for the control of the stomach emptying process. The effects of HCl concentrations and mass of contents on the emptying rates are being quantified in the model.

Publications:

Berman, M.: Compartmental Modeling. In Laughlin, J. S. and Webster, E. W. (Eds.): Advances in Medical Physics. Boston, Mass., The Second International Conference on Medical Physics, Inc., 1971, pp. 279-296.

Serial No. NIAMD, ODIR, MRB 4.2
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Iodine Kinetics

Previous Serial Number: SAME

Principal Investigator: Mones Berman

Collaborating Investigators: R. Temple, J. Wolff, J. Robbins (NIAMD), B. Belshaw, M. Barandes, D. V. Becker (Cornell Medical Center, New York)

Man Years:

Total:	.3
Professional:	.3
Others:	0

Project Description:

Objective: To model iodine kinetics and thyroid function in man and other animals.

Major Findings: Analysis of lithium effects on iodine kinetics produced several major findings: 1) lithium appears to reduce the rate of hydrolysis of thyroglobulin by a factor of about 2-3, and 2) there is a definite, previously unsuspected, effect of lithium on thyroid hormone degradation. The latter has subsequently been confirmed by more direct experimental procedures. Further studies of the details of the lithium action on iodine kinetics are in progress.

As a result of the lithium and other studies previously carried out on hyperthyroid patients (Dr. Becker), further details of the iodine kinetics model as it applies to hyperthyroidism have been resolved. In particular, the iodine trapping and binding mechanisms and the intrathyroidal iodine recycling has been quantified.

Publications:

Berman, M.: Iodine Kinetics. In Berson, S. et al (Eds.): Methods of Investigative and Diagnostic Endocrinology. Amsterdam, Holland, North-Holland Publishing Company, 1972, pp. 171-201.

Serial No. NIAMD, ODIR, MRB 4.3
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: SAAM Computer System

Previous Serial Number: SAME

Principal Investigator: Mones Berman

Other Investigators: Marjory F. Weiss

Man Years:

Total:	1.3
Professional:	1.3
Others:	0

Project Description:

Objectives: To develop a general purpose computer program for modeling bio-kinetic systems that may readily be used by investigators not sophisticated in mathematics or programming. The SAAM program was initially developed in 1959 and various phases have been developed and added to it since.

Major Findings: Considerable progress was made in the conversational SAAM system. Now through the use of a teletype and/or plotter, on-line simulations and conversational interactions with SAAM are possible. This activity will continue.

The conversion of the SAAM-25 program for use on the IBM 360 and CDC 6600 machines took considerably longer than expected, but this was finally finished. Much of the delay was due to further improvements, changes and corrections that had to be made in SAAM which had to be incorporated into the IBM and CDC systems (M. Weiss).

Serial No. NIAMD, ODIR, MRB-5
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Mathematical analysis of kinetic problems in biochemistry

Previous Serial Number: SAME

Principal Investigator: Wayne P. London

Man Years:

Total:	.5
Professional:	.5
Others:	0

Project Description:

Objectives: To develop mathematical models of the kinetics of biochemical reactions.

Major Findings: Detailed kinetic studies of the conversion of prothrombin to thrombin were designed and analyzed in collaboration with N. R. Shulman (NIAMD-CH).

When the conversion is carried out in a sizable but restricted range (called the linear range) of concentrations of prothrombin (P), activated factor ten (X_a) and soybean trysin inhibitor (S), the following properties are observed:

(i) For varying initial concentrations of P the yield curve are constant multiples of each other. In particular, the yield of thrombin (T) at any time is proportional to the initial concentration of P.

(ii) The yield curve from P added at time zero and at time τ is the sum of the yield curves of P added at time zero and the yield curve of P added at time zero but translated by time τ. Properties (i) and (ii) obtain for both uninhibited curves and inhibited curves.

(iii) The degree of inhibition produced by a given concentration of the inhibitor S is independent of the initial concentration of P. The degree of inhibition increases as the rate of conversion of P to T is decreased by lowering the concentration of X_a. S decreases both the rate of production and the final yield of T.

(iv) For a yield curve obtained with a given concentration of S, an initial concentration of P can be found such that inhibited and uninhibited curves are superimposable.

(v) If S is absent initially, but is added during the conversion of P to T, the degree of inhibition increases with the time of addition of S.

Properties (i) and (ii) imply that for P, S and X_a in the linear range, the conversion is described by linear first order differential equations with constant coefficients, the dependent variables being prothrombin, its derivatives, and thrombin.

Properties (i) and (ii) as well as other experiments exclude the possibility, suggested in literature, that the conversion of prothrombin is catalized by thrombin itself.

The yield curves were statistically fitted to the function

$$T(t) = a - be^{-\lambda t},$$

where a and b depend on the concentrations of P and S; λ was shown to be independent of S, which is the formal restatement of property (iv).

The asymptotic yields of T as a function of S were statistically fitted to the function

$$T(\infty) = \frac{P_o}{1 + a S},$$

where $T(\infty)$ is the final yield of thrombin and P_o, the initial concentration of prothrombin. This is the formal restatement of property (iii).

The simplest explanation of property (v) is that at least one intermediate between P and T has thrombin activity. Indeed, for the models cited below this assumption is necessary to explain property (v).

The simplest model consistent with all of the above findings is

$$P \xrightarrow{k} X \xrightarrow{\alpha} T$$
$$\downarrow rS$$

where $k \sim .231$/minute, $r/\alpha \sim .45$/minute and α/k is probably greater than one but less than three. The intermediate X has .5-1 times the thrombin activity per mole as thrombin. Specific experiments imply that factor X_a acts on both the $P \rightarrow X$ and the $X \rightarrow T$ steps. Other models with one intermediate and where S acts at one site are inconsistent with the data.

The following model, which is more complicated than the above model, was also considered:

$$P \xrightarrow{k} X \xrightarrow{\alpha} Y \xrightarrow{\beta} T$$
$$\downarrow r_1 S \qquad \downarrow r_1 S$$

In this case the data imply that significant inhibition by S occurs at only one site (which is essentially the simple model), that $k < \alpha$ and $k < \beta$, and that either X or Y or both have significant thrombin activity. The data are not extensive enough to permit the selection of this more complicated model instead of the simplest model.

Proposed Course: After publication of these findings, this project will be terminated.

Publications:

London, W. P. and Hearon, J. Z.: Estimation of the number of precursors in a sequence of first order reactions. Math. Biosci. (In Press).

Hearon, J. Z. and London, W. P.: Path lengths and initial derivatives in arbitrary and Hessenberg compartmental systems. Math. Biosci. (In Press).

Serial No. NIAMD, ODIR, MRB 5.1
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Mathematical analysis of the dynamics of infectious diseases

Previous Serial Number: NONE

Principal Investigator: Wayne P. London

Cooperating Units: Professor James A. Yorke
Institute for Fluid Dynamics and Applied Mathematics
University of Maryland

Man Years:

Total:	.5
Professional:	.5
Others:	0

Project Description:

Objectives: To develop mathematical models of the dynamics of infectious diseases in large populations.

Major Findings: Recurrent epidemics of infectious diseases--measles, mumps, and chicken pox--were studied using ordinary differential and differential delay equations.

These common infectious diseases are spread from individual to individual by direct contact, have incubation periods of 10-20 days, and are characterized by permanent immunity. We model the spread of infectious diseases in this class by

$$\xrightarrow{\gamma} S \rightarrow E \rightarrow I \rightarrow$$

where γ is the net input of susceptibles (S), E are the incubators--the exposed but not infectious--and I are the infectives. The mean time in the E state is the mean incubation period, and the mean time in the infectious state is the time from the onset of substantial infectivity to the time of being effectively quarantined from spreading the disease in society due to the severity of the illness, or the appearance of the characteristic rash or parotid gland swelling.

In every model we take the rate of exposure (S→E) to be

$$\beta(t)S(t)I(t)[1-cI(t)]$$

where S(t) and I(t) are the number of susceptibles and infectious at time t, respectively, and $\beta(t)$ is the fraction of the total population contacted per day by an infective. The term $-cI(t)$ is a correction term, discussed below, that is necessary at the peak of the epidemics of measles.

To model the steps E → I → we use either a system of ordinary differential equations

$$\begin{aligned} dE_1/dt &= \beta S(t)I(t) - \theta E_1 \\ dE_i/dt &= \theta[E_{i-1} - E_i] \qquad i=2,\ldots,n \\ dI/dt &= \theta E_n - \delta I, \end{aligned}$$

where n may vary, the mean time in the exposed states, $E_1,\ldots,E_n$, is the mean incubation period, and the mean time in the infectious state is about 2 days. Alternatively, we use a delay differential equation:

$$dI/dt = \beta(t)[S(t-a)I(t-a) - S(t-a-b)I(t-a-b)]$$

where a is the incubation period and b, the period of infectivity.

Computer simulations of the equations from all of these models clearly show that the recurrent epidemics that are observed every one, two or perhaps three years are not obtained unless, β, the number of contacts per day, is low during the summer and high during the rest of the year. For the ordinary differential equation model that has no incubation period, this result can be proved by finding an appropriate Liapunov function.

The reported number of cases by month for the past 40-50 years were obtained for measles in New York City and Baltimore and chicken pox and mumps in New York City.

Using the ordinary differential or the differential delay equations we calculated a β for each month such that the calculated number of monthly cases equals the reported number of cases. The calculated monthly β's (averages of about 35 years) are about 50% lower during the summer months than during the rest of the year. As long as the mean incubation period is about 12 days, both the ordinary differential equations and the delay equations give the same values of β. For the measles data, regardless of the model, the correction term in the rate of exposure, $-cI(t)$, is necessary; otherwise, at the peak of the measles epidemics, the β from the years with a large number of cases are statistically lower than the β from the years with fewer cases.

When the different models with the calculated β's are used to simulate the recurrent epidemics, all the models predict recurrent epidemics, but the delay equations most faithfully reproduce the data.

An important question is what accounts for the number of effective contacts per day, β, being about 50% lower in the summer months than during the rest of the year? Any factor that enhances the spread of the disease--the gathering of the children in school, decreased resistance to disease, longer survival of virus in cool, dry air, etc.--would yield a β that is higher in the fall, winter and spring months. Although we can not separate all the possibilities, several lines of evidence, including the points raised below, suggest that the β reflects the sociologic patterns of the susceptibles and infectives, and that the gathering of the children in school must be considered in accounting for the seasonal variation in the number of contacts.

All the models assume random mixing of susceptibles and infectives and although these models reproduce the data, there are two instances where random mixing probably does not occur:

(i) At the peak of the measles epidemics it is necessary to correct the exposure rate with the term $-cI(t)$; the observed exposure rate is less than that predicted by $\beta S(t)I(t)$, and apparently not enough susceptibles are coming into contact with the very large number of infectives.

(ii) With chicken pox in New York City the values of β from the early years (1930-1945) differ statistically from the β values of the latter years (1946-1960). The same result is found for Manhattan and Brooklyn and probably the Bronx. Again, it appears that susceptibles are not coming into contact with infectives, which may be due to the isolation of groups of people into geographic "ghettos" that was common in the boroughs in New York City during the 1930's. Why this apparent lack of mixing of infectives and susceptibles is seen only with chicken pox and not with measles or mumps is not known.

Proposed Course: After publication of these findings, this project will be terminated.

ANNUAL REPORT OF CLINICAL INVESTIGATIONS, NIAMD

July 1, 1971 to June 30, 1972

Dr. Robert S. Gordon, Jr., Clinical Director

- - -

There have been no major changes in the organizational or staffing pattern of NIAMD Clinical Investigations during FY 1972. Planning for the institution of a research unit devoted to liver diseases is under way, but it is not likely that it will begin actual work until late in FY 1973 or early in 1974.

Data on inpatient admissions and outpatient visits for the period April 1, 1971 through March 31, 1972 are as follows. 447 patients were admitted to the three nursing units under the care of NIAMD research physicians, for a total of 12,841 hospital days. The average length of stay was 28 days, and overall bed occupancy averaged 53%. During the same twelve-month period, there were 2,688 outpatient visits to the clinics of this Institute.

The average bed occupancy was noticeably lower this year than last, due to a sharp decrease in the average length of stay for each patient. (It should be noted that the total number of admissions exceeds that of last year.) To offset the low occupancy in part, NIAMD agreed to accommodate two more beds for the care of dental patients within its nursing units, freeing two beds on the seventh floor which are being incorporated into the coronary care unit of NHLI. Plans are also being forwarded to convert one patient room into a much-needed fluoroscopy, endoscopy, and procedure room for the gastroenterology service. With these changes, and with the added load expected when the liver disease service commences to recruit patients, we anticipate an increase in average occupancy in the near future.

Using the computerized file of admitting data for new NIAMD patients, we have again tabulated the distribution of admissions among our several nursing units, among the Institute's Branches, and among the more common diagnostic entities under study. The following tables may be of interest:

Admissions by Nursing Unit

9 East	171
9 West	150
8 West	126
Total	447

Admissions by Branch		Assigned Beds	Admissions per Bed	Clinical Associates on Duty
ARB	139	15	9.3	7
DHDB	60	11	5.5	3
CEB	108	14	7.7	5
CHB	36	9	4.0	2
MDB	54	10	5.4	3
PMB	50	7	7.1	2
Total	447	66	6.8	22

Admissions by Admitting Diagnosis	
Acromegaly	20
Cystic fibrosis	41
Diabetes mellitus	18
Hyperparathyroidism	27
Hyperthyroidism	8
Hypogammaglobulinemia	8
Hypoglycemia	13
Idiopathic thrombocytopenic purpura	8
Malabsorption	19
Normal volunteer	41
Rheumatoid arthritis	35
Systemic lupus erythematosus	71
Thyroid neoplasms	12
All others	126
Total	447

The Institute is still without its senior gastroenterologist, Dr. Leonard Laster, who has continued his temporary assignment with the science advisory staff at the White House. Dr. Jerry Gardner, a senior staff fellow in the Branch, has done an able job filling in for Dr. Laster, but Dr. Laster's continued absence is felt. During 1972, there have been 22 Clinical Associates on duty with the several branches of NIAMD, which have also been host to 6 Visiting Scientists and 24 Guest Workers, the latter two categories representing 6 foreign countries as well as the United States.

Studies done within the Branch in the past year can be considered under the following five headings: rheumatoid arthritis, systemic lupus erythematosus, immune cell function, immunoglobulin structure, and marker compound metabolism.

1. Rheumatoid Arthritis

The rheumatoid-like, chronically active polyarthritis of swine which can be induced in young animals by the intraperitoneal innoculation of Mycoplasma hyorhinis has been studied in detail over an 18 month course. The organism, easily recovered in the first few months, was not grown out from any of a great variety of specimens after the third month. Despite this, high antibody levels were induced and sustained into the 11th month when they began to drop. This behavior was explained by the finding of a persistent synovial M. hyorhinis antigen. The antigen was readily and specifically identified in cryostat prepared tissues using a fluorescein labeled anti-M. hyorhinis antibody. The synovial antigen decayed in amount from the 11th to the 18th month when it disappeared entirely from the last surviving animal. The destructive synovitis, with its heavy load of immunocompetent cells looking much like rheumatoid arthritis, can be regarded as the body's effort to eliminate the foreign antigen (Decker, Barden (not NIH), Hopps (DBS), Dalgard (not NIH), Ennis and Johnson (I)). Surgical removal of the inflamed soft tissue in human disease may ameliorate bone and cartilage destruction (Decker, Aptekar, Peterson (not NIH) and Fried (CC)).

2. Systemic Lupus Erythematosus

Interest in the cell type (bone marrow or thymus derived?) involved in human SLE has been stimulated by findings in the NZB/NZW mouse disease. Work with the survival of skin allografts using administered cells of various origins and various ages has neatly shown that the delayed rejection characteristic of the old B/W hybrid animal can be returned to normal by the injection of young spleen cells. If the young spleen cell donor is pretreated with corticosteroids, the effective cell population is eliminated since transfer of a standard number of cells from such a donor does not accelerate graft injection (Steinberg, Parker, Gelfand (not NIH), Scher (not NIH), Gazdar (C), Powell (not NIH) and Chused (D)).

Azathioprine, cyclophosphamide and methylprednisolone, each at a low dose of 1.5 mg/kg/day, were tested singly and in all combinations for their ability to modify the proteinuria, the evolution of anti-DNA antibodies, the renal histological lesion, and survival in NZB/W female mice starting at 5 months of age when these parameters begin to show abnormalities. It was found that the best results (86% survival) were achieved without increased drug toxicity by the three drug combination; 10% of controls survived. Giving each drug alone at 4.5 mg/kg/day produced a one year survival rate of 33%. Intermittent cyclophosphamide treatment was superior to daily

administration (Steinberg, Kovacs and Gelfand (not NIH)).

Electron microscopic study of the white blood cell buffy coat of a series of patients with connective tissue disease has shown nests of tubuloreticular structures in the lymphocytes of about 60% of patients with systemic lupus erythematosus and polymyositis and in a lesser proportion of patients with scleroderma and Sjogren's syndrome. These structures, found earlier in the endothelial cells of kidney and skin biopsies from the same patient categories, are interpreted as strong evidence of viral infection but are believed to represent a secondary cell product rather than viral material per se. The finding opens a number of important investigative channels (Decker, Grimley (C), Hopps (DBS), Michelitch (C) and Frantz)). The finding by Farr assay of antibodies to double stranded DNA in 75% and RNA in 45% of patients with SLE further strengthens the likelihood that viral antigens are present in such patients. It was shown that the two antibody populations are distinct and may constitute a significant proportion of all antibodies present (Steinberg).

Detailed neurological and psychiatric study of SLE patients has been related to complement levels in the blood and cerebrospinal fluid (CSF). Despite the evidence of others to the contrary, we have not been able to relate clinical manifestations of central nervous system disease to CSF complement levels. The key finding was the discovery of remarkable different complement decay rates upon storage of the CSF at -45°C. The patients with active disease showed very rapid decay in contrast to normals or patients with inactive disease. The mechanism of this decay continues under study (Hadler, Frank (I), Gerwin (I), Whitaker (N), Baker (M), Donnelly (M) and Decker).

Quantitation of fibrin degradation products in the urine of patients with SLE nephritis has not provided us with the hoped for measure of renal disease activity. The levels are elevated to a great degree in patients with obviously active disease but do not relate well to the status of problem patients whose nephritis is milder (Decker, Steinberg, Gralnick (CC), Marchesi (CC) and Aptekar). Careful evaluation of a single patient with SLE and marked hyperkalemia showed that a factor (? an antibody) in his circulation had effects on the transmembrane electrolyte balance of red blood cells. As the patient improved, the factor regressed and disappeared (Hadler, Gill (H) and Gardner (A).

The major committment to drug trials in SLE nephritis has continued with 25 selected patients having been admitted to receive either azathioprine, cyclophosphamide or placebo for a ten week in-hospital trial. Six measures are repeatedly assessed through the blind trial period. The results thus far show a statistically significant advantage to the drug-treated group but this has been far from striking. Believing that the long-term efficacy is much the most significant aspect, we are making efforts to continue the patients indefinitely on the program to which they were randomly assigned (Decker, Steinberg, Hadler, Salisbury and Myers (C)). Certain theoretical considerations and the mouse evidence of cyclophosphamide efficacy upon intermittent

administration has lead us to a trial of intravenous cyclophosphamide in severe SLE of any variety which is shown to be corticosteroid resistant. Such patients are randomly assigned to receive intravenous cyclophosphamide or placebo every 3 weeks for 3 doses with repeat courses at subsequent six monthly intervals. Three patients have begun the trial (Plotz, Decker and Steinberg).

3. Function of Immune Cells

The localization of the IgE previously found on human basophils was shown to vary with temperature. At low temperatures the ferritin tag was found in linear array diffusely over the entire cell surface; when the studies were performed at room or body temperature, the ferritin was distributed assymmetrically covering a surface membrane segment one-fifth to one-half the circumference. New hybrid antibodies are now being produced in large volume (sheep and burro) with a view to correlating the distribution of cell surface IgE with the course of events during histamine release by the basophil (Metzger, Becker and Grimley (C)). Two lines of mouse mastocytoma cells in culture will be used for absorption studies trying to reduce the high titers of reaginic antibodies specifically induced in rats and mice (Metzger, Wilson (not NIH) and Jones (not NIH)). Attempts continue to enrich populations of immune cells by means of the reactivity of their surface immunoglobulins (Plotz and Seaman).

The role of delayed hypersensitivity and its relationship to disease is being studied in two systems. Lymphocytes from patients categorized with respect to serum hepatitis (acute, healed, chronic, active, etc.) are being tested by tritiated thymidine incorporation in the presence of phytohemagglutinin or the hepatitis B antigen (Plotz and Agus (A)). Secondly, evidence has been developed of lymphocyte transformation to several cardiac antigens in an animal system. Preliminary evaluation of the lymphocytes of SLE patients with myocarditis shows a similar responsiveness to cardiac antigens (Salisbury and Guss (C)).

4. Immunoglobulin Structure

It was found that a diazonium nitrobenzene compound specifically labeled the light chain Tyrosine 34 of a nitrophenyl binding γA mouse myeloma protein (Metzger and Hadler). A phosphorylcholine analog, p-diazonium phenylphosphorylcholine, was prepared and reacted with a phosphorylcholine-binding mouse myeloma protein. The major labeled peptide was isolated and sequenced. The labeled tyrosine appears to be precisely homologous to the Tyrosine 34 of the nitrophenyl binding protein, a remarkable finding since the ligands are so different. It was concluded that the tyrosine is in the combining site and serves a similar function in antibodies of diverse specificities (Metzger and Chesebro).

5. Marker Compound Metabolism

Work has gone forward on compounds either administered to or known to be present in humans; in each case interest has been directed to the role of the intestinal microflora in their metabolism. Our understanding of the metabolism of guanidinosuccinic acid (GSA), caffeic acid (CA), and azulfidine (AZ) has been advanced by the work and it has pointed up the great and largely unrecognized metabolic role of intestinal bacteria. GSA, a compound much discussed as a possible cause of "uremic symptoms", is of endogenous origin; its excretion varies with the amount of nitrogen in the diet (Goldman, Young (CC) and Milstien). The material may be partially degraded by intestinal bacteria since germfree rats excrete more GSA than do conventional animals.

CA metabolism appears to depend upon the stepwise action of a series of intestinal organisms since, of 12 bacteria isolated from the human gastrointestinal tract, none were capable of catalyzing more than one reaction in its metabolic disposition. AZ metabolism, initiated by the reduction of the azo bond, depends exclusively upon intestinal microflora and thus its exact nature bears upon which metabolites of the drug are active in human disease (Goldman, Peppercorn, Milstien, and Goldin).

Serial No. NIAMD-ARB- 1C
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Role of Infection in Rheumatoid Diseases

Previous Serial Number: NIAMD-ARB-1C

Principal Investigator: Dr. John L. Decker

Other Investigators: Dr. Jerri Barden (Hazleton Laboratories), Dr. Hope Hopps (DBS-LVI), Dr. Dan W. Dalgard (Hazleton Laboratories), Dr. Robert S. Ennis and Dr. John Johnson (I-LCI).

Cooperating Units: Hazleton Laboratories, Division of Biologic Standards, Laboratory of Viral Immunology; National Institute of Allergy and Infectious Diseases, Laboratory of Clinical Investigations.

Man Years:

Total:	1/2
Professional:	1/4
Other:	1/4

Project Description:

Objectives:

The overwhelming likelihood is that rheumatoid arthritis is due to an infection. There is a long history of failure in trying to isolate the responsible microbial agent. We therefore believe it important to study the biology of the host-parasite relationship using a known microbial agent to produce an animal disease similar to rheumatoid arthritis. To this purpose we have been assessing the events in the evolution of chronic Mycoplasma hyorhinis arthritis of swine.

Methods Employed:

The polyarthritis which develops following intraperitoneal injection of Mycoplasma hyorhinis has been studied over an 18 month period by: repeated clinical evaluation; serial determination of serum proteins and of serum antibody activity to M. hyorhinis; serial blood cultures; sequential autopsies with material for culture, tissue culture, immunofluorescent studies, and histopathology; sequential synovial fluids for antibody activity.

Serial No. NIAMD-ARB-2C
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on Swine Gamma Globulins

Previous Serial Number: NIAMD-ARB-2C

Principal Investigator: Dr. John L. Decker

Other Investigators: Dr. John S. Johnson (I-LCI), Dr. H. Benfer Kaltreider (Univ. of California at San Francisco) and Dr. Jerri Barden (Hazleton Laboratories).

Cooperating Units: National Institute of Allergy and Infectious Diseases, University of California at San Francisco and Hazleton Laboratories, Falls Church, Virginia.

Man Years:

Total:	0
Professional:	0
Other:	0

Project Description:

This project has been completed and the results are reported under Project Serial Number NIAMD-ARB-1C (July 1, 1970 through June 30, 1971), Role of Infection in Rheumatoid Diseases.

Serial No. NIAMD-ARB-3C
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Retrospective Evaluation of Knee Synovectomy in Rheumatoid Arthritis

Previous Serial Number: NIAMD-ARB-3C

Principal Investigator: Dr. John L. Decker

Other Investigators: Dr. Robert G. Aptekar, Dr. Leonard T. Peterson, Orthopedic Consultant, George Washington University School of Medicine and Dr. David Fried (CC:Rehab).

Cooperating Units: George Washington University School of Medicine, Washington, D. C. and Clinical Center, Rehabilitation Department.

Man Years:

Total:	3/4
Professional:	1/2
Other:	1/4

Project Description:

Objectives:

To discover whether or not prophylactic synovectomy, as defined below, will alter the functional or structural prognosis in rheumatoid arthritis.

Methods Employed:

Patients with rheumatoid arthritis who have had at least six months of active inflammation in both of a pair of symmetrical joints, neither of which show radiological changes other than osteoporosis, have been admitted to the study. The disease must be sufficiently symmetrical to have all parties -- the investigator, the surgeon and the patient -- agree to have either one of the pair operated. The side to be done is selected by lot.

The patients are then seen at intervals of six months, one year, and two years. On these visits they undergo standard evaluation for function and radiological examination. Extended follow-up for as long as five years has been completed.

Major Findings:

Thus far it is possible to say that, at 18 months followup, the operated knee was still preferred clinically. The radiological changes have not been impressive in either knee. Twelve patients have been studied to date. It appears that the most important determinant of successful surgery has been the general state of the arthritis.

Significance to Bio-Medical Research and the Program of the Institute:

There has been essentially no controlled work on this important therapeutic modality which is being widely urged on the basis of inadequate data. There is reason to believe that, almost alone among treatment methods, it will prevent joint damage. This needs to be established.

Proposed Course:

Prelimiary analysis of the initial group is underway.

It is proposed to continue to admit suitably selected patients to the study.

Honors and Awards: None

Publications: None

Serial No. NIAMD-ARB-4C
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Assessment of Synovial Physiologic Variables

Previous Serial Number: NIAMD-ARB-4C

Principal Investigator: Dr. Edward J. Goetzl

Other Investigators: Dr. John L. Decker, Dr. Kenneth H. Falchuk (H:LKEM), Dr. Louis S. Zeiger (Nuclear Medicine, NIH), and Dr. John P. Adams (George Washington University).

Cooperating Units: National Heart Institute, Laboratory of Kidney and Electrolyte Metabolism, Nuclear Medicine, NIH, and George Washington University School of Medicine, Department of Orthopedic Surgery.

Man Years:

Total:	0
Professional:	0
Other:	0

Project Description:

This study has been terminated because of the departure of the Principal Investigator.

Publications:

Goetzl EJ, Falchuk KH, Zeiger LS, et al: A physiological approach to the assessment of disease activity in rheumatoid arthritis. J Clin Invest 50:1167-1180, 1971

Serial No. NIAMD-ARB-5C
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Pathogenesis of Autoimmunity in New Zealand Mice

Previous Serial Number: 12C

Principal Investigator: Dr. Alfred Steinberg

Other Investigators: Dr. Leroy Parker, Dr. Michael Gelfand (Walter Reed General Hospital), Dr. Irwin Scher (NNMC), Dr. Adi Gazdar (C-VLL), Dr. Deborah Powell (Georgetown University Hospital and Dr. Thomas Chused (D-OMS).

Cooperating Units: Walter Reed General Hospital, National Navy Medical Center, NCI-VLL, Georgetown University Hospital and D-OMS Branch.

Man Years:

Total:	1-1/2
Professional:	1
Other:	1/2

Project Description:

Objectives:

New Zealand mice spontaneously develop an autoimmune disease resembling human systemic lupus erythematosus. Early in life they become relatively resistant to tolerance induction, later they spontaneously develop antibodies to RNA and DNA and finally they develop immune complex glomerulonephritis from which they die. Both humoral and cellular immune defects have been reported. Since antibodies to nucleic acids, genetic factors and viral infections appear to be important in this disease, all are being studied in New Zealand mice as well as control mice.

Our specific recent objectives have been 1) to more fully study the immunogenecity of nucleic acids, 2) study the non-antigenic properties of nucleic acids, 3) study the genetics of both autoimmunity in New Zealand mice and of responsiveness to nucleic acid immunizationa, and 4) define the cellular basis for the immunologic abnormalities of New Zealand mice.

Methods Employed:

1. Assay for antibodies to RNA and DNA using ^{14}C-labelled nucleic acids as ligands and the ammonium sulfate precipitation assay.

2. Immunization of animals with a variety of nucleic acid antigens with and without protein carriers or adjuvants.

3. Transfer of spleen bone marrow and thymus cells into irradiated or cyclophosphamide treated recipients to evaluate the functional properties of the different cell types.

4. Use of standard skin grafting using allogenic skin to evaluate cellular immune functions in New Zealand mice, and transfer of serum and cell population between young and old New Zealand mice to study the defect in old New Zealand mice.

Major Findings:

1. Double stranded RNA resembles other antigens more than was previously thought. There is a clear cut early peak (4 days) which is IgM in response to fluid rI·rC. Emulsified with adjuvant rI·rC leads to an additional late peak which is largely IgG. Requirements for protein carrier depends upon antigen dose, strain, and other factors.

2. Spontaneous antibody to nucleic acids and ability to be immunized with rI·rC are under genetic control. The response to immunization is dominant or co-dominant. In one strain at least CBA/HN the non-responder status is sex-linked.

3. Nucleic acids are rapidly metabolized in vivo. The metabolism of RNA and DNA is the same in New Zealand as in normal mice.

4. New Zealand mice do not respond well to the adjuvant properties of poly I·poly C with regard to the SRBC response, however, they do respond to the adjuvant properties with regard to antibodies to nucleic acids.

5. Skin allograft rejection was studied in young (2 month) and old (8 month) female NZB/NZW F_1 (B/W), DBA/2, and Swiss mice. Old B/W, but not DBA/2 or Swiss mice rejected C57Bl/6 skin grafts significantly more slowly than did young mice of the same strain. Splenectomy did not further prolong graft survival in old B/W mice, but did in the other groups, suggesting a deficiency in the spleen cell population of old B/W's.

Splenectomized old B/W's could be restored to "normal" by i.p. injection of large numbers of young spleen cells (50×10^6). Intact old B/W mice were restored by as few as 5×10^6 young spleen cells, suggesting cooperation between young and old spleen cell populations. This was demonstrated by

studies in splenectomized old B/W mice. The combination of 5 x 10^6 young and 100 x 10^6 old B/W spleen cells led to normal skin graft rejection, whereas neither alone was effective. This synergy required to live thymus derived cells: bone marrow derived cells or frozen and thawed spleen cells were ineffective. Pre-treatment of the young donor with corticosteroids (100 mg/kg methylprednisolone) 48 hours before obtaining the spleen cells abolished this synergy. Similar pre-treatment of the old donor did not. We conclude that a population of steroid sensitive spleen cells is lacking or ineffectual in old B/W mice. These cells are present in young B/W mice and synergize with a population of steroid resistant spleen cells in skin graft rejection.

Significance to Bio-Medical Research and the Program of the Institute:

Our studies establish the antigenic nature of double stranded RNA. They are unravelling the complex genetic factors involved in nucleic acid immunization and autoimmunity. They exclude a nucleic acid metabolic abnormality in New Zealand mice as a cause of spontaneous antibodies to nucleic acids. They define the cellular basis for the immunologic abnormalities of older New Zealand mice.

Proposed Course:

1. Study the basis for the cellular abnormality of New Zealand mice and attempt specific immunotherapy.

2. Continue to define the genetic mechanisms involved in nucleic acid immunity and determine the cellular basis for the effects.

3. Study control mechanisms for antinucleic acid antibody production in New Zealand and normal mice.

Honors and Awards: None

Publications:

1. Steinberg AD, Pincus T, Talal N: The pathogenesis of autoimmunity in New Zealand mice. III. Factors influencing the formation of antinucleic acid antibodies. Immunology 20:523-531, 1971

2. Steinberg AD, Baron S, Uhlendorf C, et al: Depression of the interferon response to polyinosinic·polycytidylic acid by specific antibody. Proc Soc Exp Biol Med 137:558-561, 1971

3. Talal N, Steinberg AD, Jacobs ME, et al: Immune cell cooperation, viruses and antibodies to nucleic acids in New Zealand mice. J Exp Med 134: 52s-64s, 1971

4. Talal N, Steinberg AD, Gazdar A: Specific immunosuppression, polyinosinic·polycytidylic acid and viruses in New Zealand mice. Fed Proc 30:1842-1845, 1971

5. van Boxel JA, Steinberg AD, Green I: A study of the cell mediated and antibody responses to synthetic ribonucleic acids in guinea pigs. J Immunol 108:466

6. Gazdar AF, Weinstein AJ, Simms HL, Steinberg AD: Enhancement and suppression of murine sarcoma virus induced tumors by polyriboinosinic·polyribocytidylic acid. Proc Soc Exp Biol Med 139:279-284, 1972

7. Gazdar AF, Steinberg AD, Spahn GJ, et al: Virus-induced sarcoma and leukemia: Enhancement in mice and rats by several interferon inducus. Proc Soc Exp Biol Med (In press)

8. Powell DE, Steinberg AD: The pathogenesis of autoimmunity in New Zealand mice. IV. Independent stimulation of antibodies to DNA and RNA. Clin Exp Immunol (In press)

9. Chused TM, Steinberg AD, Talal N: The clearance and localization of nucleic acids by New Zealand and normal mice. Clin Expt Immunol (In press)

Serial No. NIAMD-ARB-6C
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Therapeutic studies in New Zealand mice

Previous Serial Number: None

Principal Investigator: Dr. Alfred D. Steinberg

Other Investigators: Dr. Katalin Kovacs and Dr. Michael Gelfand (Walter Reed General Hospital)

Cooperating Units: Walter Reed General Hospital

Man Years:

Total:	1
Professional	3/4
Other:	1/4

Project Description:

Objectives:

New Zealand mice, especially the NZB/NZW F_1 spontaneously develop antibodies to nucleic acids and die of immune complex glomerulonephritis. These mice are an excellent model for comparing different immunosuppressive drug programs. In addition, as the immunologic basis for the disease becomes better understood, they offer a model for studying specific immunotherapy.

Methods Employed:

1. Study of a) The hematologic and toxic effects of cyclophosphamide in normal and New Zealand mice, b) the effect of bone marrow transplantation in reversing such toxicity, c) the interaction of cyclophosphamide with other drugs with which it is commonly used.

2. Comparison of different immunosuppressive drugs given daily singly or in combination upon antibodies to DNA (measured by the ammonium sulfate precipitation assay), proteinuria, kidney histology and survival. Comparison of daily versus intermittent therapy.

3. Transfer of syngenic or allogenic young thymus or spleen cells into New Zealand mice in an attempt to favorably modify this disease.

Major Findings:

Three lethal syndromes related to cyclophosphamide administration were distinguished: 1) Acute toxic death following a single large dose, 600 mg/kg. 2) The same dose divided into two injections separated by 24 hours, or a lower single dose (400 mg/kg), led to death within two weeks with the loss of circulating small lymphocytes. 3) Daily cyclophosphamide (40 mg/kg) led to profound bone marrow depression, superinfection, wasting and death. Bone marrow transplantation was of value in the last two syndromes, but was ineffective in altering the acute toxic deaths. Chloroquine (3 doses of 20 mg/kg) markedly increased cyclophosphamide toxicity. This occurred when the cyclophosphamide was given either as a single large dose (300-600 mg/kg) or in smaller daily doses (40 mg/kg). High dose prednisolone therapy (20 mg/kg/day for 8 days), but not low dose (5 mg/kg/day for 8 days), also increased the toxicity of cyclophosphamide.

Groups of 5 month old female NZB/W mice were given azathioprine, cyclophosphamide and methylprednisolone in all 1-, 2- and 3- drug regimens, each drug in the relatively low dose of 1.5 mg/kg/day. Treatment for three months with 1 or 2 drugs resulted in modest suppression of NZB/W disease. Mice receiving all three drugs had significantly less proteinuria, lower titers of anti-DNA antibody and less severe histologically evident renal involvement than mice treated with 1 or 2 drugs. Survival at one year was 10% for untreated controls, 44% for 1-drug treated, 37% for 2-drug treated and 86% for the 3-drug treated mice. The survival for the 3-drug regimen was significantly longer than any other group ($p<0.01$). The 3-drug regimen was synergistic, since mice treated with each drug at 3 times the dose had significantly more proteinuria after three months of treatment and lowered one year survival (33%). The beneficial effects of triple-drug therapy were attained without increased toxicity.

At low doses (1.5 mg/kg/day), azathioprine was most effective. Cyclophosphamide was superior at higher doses (4.5 mg/kg/day). Different two drug combinations were also compared. The combination of cyclophosphamide plus methylprednisolone was most effective. Combined therapy increased survival without increasing toxicity.

Intermittent cyclophosphamide therapy was superior to daily therapy.

Significance to Bio-Medical Research and the Program of the Institute:

These studies have paved the way for clinical studies in which the immunosuppressive drugs may be given in a more effective manner.

Proposed Course:

Continued attempt at specific immunological therapy in coordination with the pathogenetic studies we have been carrying out.

Publications:

1. Kovacs K, Steinberg AD: Cyclophosphamide: Drug interactions and bone marrow transplantation. Transplantation 13:316-321, 1972

2. Steinberg AD, Plotz PH, Wolff SM, et al: Cytotoxic drugs in treatment of non-malignant diseases. Ann Intern Med 76:619-642, 1972

3. Gelfand MC, Steinberg AD, Nagle R, et al: Therapeutic studies in NZB/W mice. I. Synergy of azathioprine, cyclophosphamide and methylprednisolone in combination. Arthritis Rheum 15:239-246, 1972

4. Gelfand MC, Steinberg AD: Therapeutic studies in NZB/W mice. II. Relative efficacy of azathioprine, cyclophosphamide and methylprednisolone. Arthritis Rheum 15:247-252, 1972

Serial No. NIAMD-ARB- 7C
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Tubuloreticular Structures of Circulating Lymphocytes in Systemic Lupus Erythematosus

Previous Serial Number: None

Principle Investigator: Dr. John L. Decker

Other Investigators: Dr. Philip M. Grimley (NCI), Dr. Hope Hopps (DBS), Mr. Herman Michelitch (NCI) and Mrs. Molly Frantz

Cooperating Units: National Cancer Institute, Laboratory of Pathology; and Division of Biologics Standards, Laboratory of Viral Immunology

Man Years:

Total:	3/4
Professional:	1/4
Other:	1/2

Project Description:

Objectives:

Nests of interwoven tubuloreticular structures (TRS) have been recognized by electron microscopy in the cytoplasm of the endothelial cells of kidney and skin from patients with systemic lupus erythematosus for several years now. TRS, also identified in endothelial cells from cases with Sjogren's syndrome, polymyositis and scleroderma, are considered by some to be paramyxovirus particles but others, finding them associated with a number of experimental viral infections, interpret them as nonspecific cellular response to viral infection.

Upon examining a skin biopsy specimen from one of our patients with SLE, TRS were seen not only in the endothelium as expected but also within the cytoplasm of a circulating mononuclear cell, probably a lymphocyte.

The objective of study was to assess the prevalence of TRS in buffy coat lymphocytes from patients with connective tissue disease. This objective is consonant with the larger objective of understanding the pathogenesis of SLE and related diseases.

Methods Employed:

A specifically selected group of patients and normal controls were bled and buffy coats were prepared, fixed, and thin sectioned. At least one hundred mononuclear cell sections from each buffy coat were scanned. The microscopist was unaware of the source of the first 90 specimens examined.

Major Findings:

1. TRS was found in the lymphocytes from 20 of 30 patients with SLE and in a smaller proportion of patients with discoid LE, polymyositis, scleroderma and Sjogren's syndrome.

2. TRS has not been identified in normal individuals nor in patients with rheumatoid arthritis or infectious mononucleosis.

3. A comparison of a number of clinical and laboratory measures between 20 SLE patients with TRS and 10 SLE patients without TRS has identified no direct correlates but suggests that the structures are most likely to appear when the disease is most active.

Significance to Bio-Medical Research and the Program of the Institute:

The finding is of considerable interest in spanning two disparate cell types, circulating mononuclear cells and endothelial cells, both sites of disease in SLE. The TRS appear to be membrane bound by the endoplasmic reticulin and are presumably generated by the cell itself and not phagocytosed. The finding of the material in readily sampled buffy coats will permit more rapid progress in its comprehension than could be expected with tissue biopsy sampling modes.

Proposed Course:

Active study of the phenomenon continues as follows: 1) using various buffy coat techniques to identify one producing the best yields of unmixed lymphocytes; 2) getting more patients examined especially those with rheumatoid arthritis (RA) since the TRS difference between RA and SLE is remarkable indeed; 3) staining of LE buffy coats with ferritin-labelled anti-Ig antibodies permitting a conclusion on whether bone marrow or thymus derived lymphocytes carry the TRS; 4) attempts to develop a permanent cell culture line carrying TRS from SLE buffy coats.

Honors and Awards: None

Publications: None

Serial No. NIAMD-ARB-8C
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Antibodies to Double Stranded DNA and RNA in Spontaneous and Drug-Induced Systemic Lupus Erythematosus and other Diseases

Previous Serial Number: 13C

Principal Investigator: Dr. Alfred Steinberg

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1/2
Professional:	1/4
Other:	1/4

Project Description:

Objectives:

Study the specificity of anti-DNA and anti-RNA antibodies in human and murine lupus and their frequency in other diseases.

Methods Employed:

Farr ammonium sulfate precipitation assay, using single or double stranded C^{14}-KB cell DNA, or C^{14} polyinosinic·polycytidylic acid as antigen. Standard precipitin reaction using unlabelled nucleic acids.

Major Findings:

1. Antibodies to double stranded DNA and RNA are found respectively in 75% and 45% of human lupus patients, and rarely in other human autoimmune disorders, including drug-induced lupus.

2. Inhibition studies demonstrate that these are largely distinct antibody populations that may be directed against viral nucleic acids. Antibodies to RNA have specificity for several double-stranded RNAs investigated. Such antibodies arising spontaneously in B/W mice seem to have greater affinity for RNA than similar antibodies appearing in human lupus.

3. Antibodies to DNA and RNA were quantitated and found to represent a significant percentage of all antibody in certain instances.

Significance to Bio-Medical Research and the Program of the Institute:

The presence of antibodies to double-stranded DNA and RNA strengthens the possibility of viral antigens in human lupus.

Proposed Course:

Continued clinical study of anti-DNA and anti-RNA antibodies in SLE and other diseases. Study of the specificity of antibodies to nucleic acids in drug-induced SLE. In view of the relationship between Australia antigen and connective tissue disease and between SLE and chronic hepatitis, antibodies to nucleic acids in hepatitis (before, during and after) will be studied.

Honors and Awards: None

Publications:

1. Schur PH, Stollar BD, Steinberg AD, et al: Incidence of antibodies to double-stranded RNA in systemic lupus erythematosus and related diseases. Arthritis Rheum 14:342-347, 1971

2. Talal N, Steinberg AD, Daley G: Inhibition of antibodies binding polyinosinic·polycytidylic acid in human and mouse lupus sera by viral and synthetic ribonucleic acids. J Clin Invest 50:1248-1252, 1971

3. Steinberg AD, Kaltreider HB, Staples PJ et al: Cyclophosphamide in lupus nephritis: A controlled trial. Ann Intern Med 75:165-171, 1971

4. Steinberg AD, Pincus T, Talal N: The pathogenesis of autoimmunity in New Zealand mice. III. Factors influencing the formation of antinucleic acid antibodies. Immunology 20:523-531, 1971

Serial No. NIAMD-ARB- 9C
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on the pathophysiology of nervous system involvement in Systemic Lupus Erythematosus (SLE)

Previous Serial Number: None

Principal Investigator: Dr. Nortin M. Hadler

Other Investigators: Dr. Michael Frank (I:LCI), Dr. Robert Gerwin (I:LCI), Dr. John Whitaker (N:IR), Dr. Mac Baker (M:DCBR), Dr. Edward Donnelly (M:AP) and Dr. John L. Decker

Cooperating Units: National Institute of Allergy and Infectious Diseases, Laboratory of Clinical Investigations; National Institute of Neurological Diseases and Stroke, Intramural Research; National Institute of Mental Health, Division of Clinical and Behavioral Research

Man Years:

Total:	3/4
Professional:	1/2
Other:	1/4

Project Description:

Objectives:

To attempt to relate the neuropsychiatric crises of hospitalized patients with SLE to the hemolytic titer of the fourth component of complement (C'4) in the cerebrospinal fluid (CSF).

Methods Employed:

C'4 is titered in fresh CSF (and concurrently sampled serum) samples, utilizing C'4-deficient guinea pig sera in a hemolytic molecular assay. Lumbar punctures are performed at the start and, when possible, at termination of hospitalization and at times during the course when a change in neuropsychiatric status is noted. Patients are evaluated initially and serially by a neurologist and psychiatrist, and subjected to a thorough battery of psychological testing initially and after 10 weeks of in hospital evaluation.

Major Findings:

1. Serial CSF C'4 titers can be used retrospectively to confirm the clinical diagnosis of CNS-lupus but single CSF C'4 values do not discriminate neuro-psychiatric involvement.

2. Patients with SLE can be divided into two subpopulations based on the rate of decay of CSF C'4 stored at -45°C. The "fast" decay rate group is distinctive for the presence of active renal disease.

3. Psychiatric illness does not correlate with the severity of illness, neurologic involvement or renal impairment. Constant low dose corticosteroid treatment cannot be implicated as causative of psychiatric illness in SLE.

Significance to Bio-Medical Research and the Program of the Institute:

This is the first systematic longitudinal study of neuro-psychiatric involvement in SLE. It implicates immune mechanisms in the pathophysiology.

Proposed Course:

Study is being terminated. Further investigation will be directed toward the mechanism of CSF C'4 decay.

Honors and Awards: None

Publications: None

Serial No. NIAMD-ARB-10C
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Systemic Lupus Erythematosus Coagulation Study

Previous Serial Number: NIAMD-ARB-8c

Principal Investigator: Dr. John L. Decker

Other Investigators: Dr. Alfred D. Steinberg, Dr. Harvey Gralnick (CC-CP), Dr. Sally Marchesi (CC-CH), and Dr. Robert Aptekar

Cooperating Units: Clinical Center, Clinical Pathology Department

Man Years:

Total:	1
Professional:	3/4
Other:	1/4

Project Description:

Objectives:

To study abnormalities of coagulation in patients with systemic lupus erythematosus (SLE), and to correlate these abnormalities with disease activity.

Methods Employed:

Patients with SLE who are admitted to the Clinical Center and selected patients with SLE seen in the out-patient department are studied.

Evaluation of clinical status is done independently by Drs. Decker and Steinberg and the results recorded on separate evaluation sheets. On the same day, the patient's blood is studied for coagulation factors, fibrin split products, and other hematological parameters. A 24-hour urine is also studied for fibrin split products. The coagulation study by clinical hematology is performed without knowledge of the clinical evaluation.

Major Findings: None

Significance to Bio-Medical Research and the Program of the Institute:

This study complements nicely our other interests in SLE. It is hoped that the study will define the usefulness of fibrin split products as an index of disease activity independent of our usual immunological parameters. In addition, the study may provide further insights into the pathophysiology of SLE.

Proposed Course:

Serial study over the next year of patients on whom data has been obtained.

Honors and Awards: None

Publications: None

Serial No. NIAMD-ARB-11C
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Cation Transport Defects in SLE

Previous Serial Number: None

Principal Investigator: Dr. Nortin M. Hadler

Other Investigators: Dr. John R. Gill (H:CE), and Dr. Jerry Gardner (A:D + HD)

Cooperating Units: National Heart and Lung Institute, Endocrinology Branch; National Institute of Arthrits and Metabolic Diseases, Digestive & Hereditary Diseases Branch

Man Years:

Total:	1/2
Professional:	1/4
Other:	1/4

Project Description:

Objectives:

Patient DL NIH# 08-70-67, a 23 year old, male with Systemic Lupus Erythematosus, presented with hyperkalemia in the face of minimal impairment in creatinine clearance. These studies were undertaken to elucidate the pathophysiology.

Methods Employed:

Metabolic balance studies were undertaken in Clinical Center. Sweat electrolytes were quantitated following iontophoresis and thermal induction. Erythrocyte sodium fluxes were quantitated using isotopic tracer techniques.

Major Findings:

1. Defective renal distal tubular secretion of K^+ and poor sweat duct reabsorption of NaCl was demonstrated.

2. The patients's plasma inhibited erythrocyte sodium outflux in normal erythrocytes.

Significance to Bio-Medical Research and the Program of the Institute:

The results suggest the presence of circulating factors that can effect three distinct cation transport mechanisms simultaneously. The implication is that the factors effect components shared by all mechanisms. The work supports the existence of a K^+ secretory mechanism in the distal tubule in man.

The situation seems to represent another example of acquired circulating factors causing a clinical syndrome in SLE.

Proposed Course:

Studies are being devised to characterize the plasma factor.

Honors and Awards: None

Publications:

Hadler NM, Gill JR, Jr., Gardner JD: Impaired Renal Tubular Secretion of Potassium, Elevated Sweat Sodium Chloride Concentration and Plasma Inhibition of Erythrocyte Sodium Outflux as complications of Systemic Lupus Erythrocyte. Arth Rheum (in press).

Serial No. NIAMD-ARB-12C
1. Arthritis and Rheumatism
2. Connective Tissue Diseases
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Controlled study of cyclophosphamide and azathioprine in systemic lupus erythematosus with nephritis

Previous Serial Number: 7c

Principal Investigators: Dr. John L. Decker and Dr. Alfred D. Steinberg

Other Investigators: Dr. Nortin M. Hadler, Dr. Kent Salisbury and Dr. George Myers (C-Surg)

Cooperating Units: National Cancer Institute, Surgery Branch

Man Years:

Total:	1
Professional:	3/4
Other:	1/4

Project Description:

Objectives:

An earlier study (Serial No. ARB-6c, 1971) has demonstrated that cyclophosphamide has a substantial effect on systemic lupus erythematosus (SLE). Reductions of anti-DNA antibodies and increases in serum complement values were more impressive than was amelioration of renal disease. Azathioprine is being widely used by others in SLE. Others have shown that cyclophosphamide has substantially more effect on NZB/W mouse renal disease, a model of lupus nephritis, than does azathioprine.

In view of the significant toxicity of both drugs, it is important to compare them directly within a single trial. The objectives are to assess the therapeutic efficacy/toxicity of azathioprine and cyclophosphamide in comparison to each other and to placebo in SLE nephritis.

Methods Employed:

About 40 patients meeting predefined criteria for diagnosis and activity of nephritis will be admitted to the 12 week trial. In the two week baseline control period, the patients will be kept on the anti-inflammatory regimen (usually prednisone and aspirin) needed to control extra-renal manifestations. These will be continued into the ten week study period with as little change

as possible and with the addition of randomly assigned cyclophosphamide, azathioprine or placebo. Dosage of each drug will be 3-4 mg/kg/day unless leukopenia (less than 3,000 WBC/mm^3) requires reduction below that level.

A large number of serological, chemical, and urine changes will be followed in the course. Immunoglobulin levels, anti-DNA antibodies, serum complement (by hemolytic and by precipitin assay), urinary fibrin split products, and the response to a number of antigens (for delayed hypersensitivity and circulating antibodies) are among those parameters.

Major Findings:

Twenty-five patients (23 females and 2 males) with systemic lupus erythematosus, diffuse glomerulonephritis on biopsy, and clinically active renal disease have completed the trial. Patients randomly received azathioprine (AZ), cyclophosphamide (CY) or placebo (PL). Concurrent corticosteroid therapy up to 30 mg/day prednisone was permitted for control of extra-renal symptoms. This averaged in mg/day 17.9 for AZ patients, 19.7 for CY and 23.4 for PL, and was held steady. The table lists the number of patients showing improvement (+) or deterioration (-) in each measure for each treatment during the 10 weeks. Net change indicates [number improved (+) - number worsened (-)]/total measures.

	AZ(6)		CY(8)		PL(11)	
Measure	+	-	+	-	+	-
Creatinine Clearance	3	2	3	1	4	3
Urine sediment	2	2	6	0	3	4
Proteinuria	1	0	2	1	0	5
Serum Complement	1	1	3	0	2	1
Anti-DNA antibodies	4	0	4	0	2	0
Extra-renal disease	0	1	0	0	0	6
Net Change	+5/36		+16/48		-8/66	

CY (average dose 1.66 mg/kg/day) preferentially reduced lymphocytes, whereas AZ (average dose 1.93 mg/kg/day) preferentially reduced neutrophils. Both (CY, AZ) reduced IgM more than IgG or IgA. During the 10 weeks, two PL patients were withdrawn because of deterioration and 1 AZ for drug allergy. To date 4 patients have died: 1 CY of pneumocystitis carinii; 2 PL of intracranial hemorrhage during renal deterioration; 1 PL of pneumonia during subsequent AZ treatment. We conclude that both AZ and CY were superior to PL during the 10 week study. Long-term data is not yet sufficient for a choice between treatments.

Significance to Bio-Medical Research and the Program of the Institute:

If differences in the response of the disease and some of the immunological processes can be detected between the alkylating agent and the purine analogue, it will be possible to draw conclusions in reference to the cell

populations involved in systemic lupus erythematosus and thus extend our understanding of the disease process itself. It is expected that the results will permit us to select between the two in ordinary clinical management.

Proposed Course:

The study will be carried through as outlined until 40 patients complete the trial. Thereafter, a long-term followup of these patients will take place by one-week admissions every 6 months.

Honors and Awards: None

Publications: None

Serial No. NIAMD-ARB-13C
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Infection in Systemic Lupus Erythematosus

Previous Serial Number: NIAMD-ARB-9C

Principal Investigator: Dr. John L. Decker

Other Investigators: Dr. Parker J. Staples (Strong Memorial Hospital), Dr. Robert S. Gordon (A-CI), and Dr. Dale Gerding (CR-OD)

Cooperating Units: Strong Memorial Hospital, Rochester, New York; NIAMD, Clinical Investigations; Division of Computer Research and Technology, Office of the Director

Man Years:

Total:	1/2
Professional:	1/4
Other:	1/4

Project Description:

The assemblage of data has been completed.

Proposed Course:

The report detailing the findings is currently in revision.

Serial No. NIAMD-ARB -14C
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Therapy of SLE: A controlled trial comparing high dose intravenous cyclophosphamide with placebo.

Previous Serial Number: None

Principal Investigator: Dr. Paul H. Plotz

Other Investigators: Dr. John L. Decker
Dr. Alfred D. Steinberg

Cooperating Units: None

Man Years:

Total:	1
Professional:	1/2
Other:	1/2

Project Description:

Objectives:

At present, no satisfactory therapy exists for severe manifestation of SLE except for renal disease. Even renal disease does not respond well to conventional therapy when the creatinine clearance is markedly reduced. In this study we will test the efficacy of high dose intravenous therapy with cyclophosphamide for the control of severe SLE unresponsive to a trial of high dose of prednisone (up to 2 mg per kg for 3 weeks).

Methods Employed:

Patients with 1) severe renal disease or rapid deterioration of renal function 2) sensitivity to azathioprine precluding the CAP protocol 3) severe extra-renal lupus (seizures, hemorrhage, psychosis, hemolytic anemia, myocarditis, or pericarditis uncontrolled by high-dose prednisone) will be randomly assigned to receive intravenous cyclophosphamide or placebo for 3 initial injections over 6 weeks and then at 3 month intervals. Renal and non-renal patients will be separately randomized. The study will be double-blind: neither physicians nor patients will know which therapy is being administered. The end point of the study will be death or the need for renal dialysis except that patients well for 1 year may have therapy stopped. The potential hazards and benefits will be explained in full to all patients

or their families.

Major Findings:

Three patients have been admitted to the study so far - one in the non-renal group and two in the renal group. No findings of significance to date.

Significance to Bio-Medical Research and the Program of the Institute:

The therapy of SLE has been under study here for several years. The present study is an attempt to extend the therapy for patients unresponsive to conventional therapy.

Proposed Course:

We plan to enter 20 to 30 patients into the study over the next 3-5 years.

Honors and Awards: None

Publications: None

Serial No. NIAMD-ARB-15C
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Is tolerance in mice due to opsonization of antigen-sensitive cells?

Previous Serial Number: None

Principal Investigator: Dr. Paul H. Plotz

Other Investigators: Mrs. Kay Gordon

Cooperating Units: None

Man Years:

Total:	1/2
Professional:	1/4
Other:	1/4

Project Description:

Objectives:

The mechanisms involved in the induction of tolerance are obscure. One view holds that cells exposed to antigen in certain conditions are simply turned off but remain present. Another view holds that antigen causes the disappearance of antigen-sensitive cells by an unknown mechanism. These experiments were designed to determine whether the presence of antigen on the surface of an antigen-sensitive cell would render it sensitive to destruction by macrophages a) without antibody present, b) with antibody present, c) with antibody and complement present.

Methods Employed:

In an homologous system, spleen cells from unprimed or primed mice were exposed successively to antigen, antibody, complement, and active macrophages to determine if they would lose their ability to transfer an immune response to irradiated recipients.

Major Findings:

In an extensive series of experiments with several antigens, various kinds of antibody and under various conditions, we have been unable to deplete the ability of cells to respond on subsequent transfer.

Proposed Course:

After completing a small series of experiments to round out this work, it will be discontinued.

Significance to Bio-Medical Research and the Program of the Institute:

Understanding tolerance and its breakdown are critical to understanding the development of auto-antibodies. Unfortunately, these negative results do not establish a plausible mechanism for tolerance induction.

Honors and Awards: None

Publications: None

Serial No. NIAMD-ARB-16C
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Leukopenia in Connective Tissue Disease

Previous Serial Number: NIAMD-ARB-10C

Principal Investigator: Dr. John L. Decker

Other Investigators: Dr. Sheldon Wolff (I-LCI) and Dr. Harry Kimball (I-LCI)

Cooperating Units: National Institute of Allergy and Infectious Diseases, Laboratory of Clinical Investigation

Proposed Course:

The project has been set aside for the moment because of lack of specific type of patients.

Serial No. NIAMD-ARB-17C
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Immunoglobulins on Cell Surfaces

Previous Serial Number: 15c

Principal Investigator: Dr. Henry Metzger

Other Investigators: Dr. Karl Becker and Dr. Philip M. Grimley

Cooperating Units: Section on Pathological Anatomy, NCI

Man Years:

Total:	2
Professional:	1-1/4
Other:	3/4

Project Description:

Objectives:

Essentially all the important activities of antibodies are ultimately expressed not in free solution but on cell surfaces. For example antibodies act as antigen receptors on cells destined to respond in the immune response. Similarly it is γE on effector cells which when stimulate by allergens, leads to histamine release. Specific antigen catabolism similarly must be mediated by antigen antibody complexes interacting with phagocytic cells. We are now establishing a variety of experimental situations whereby we can look at such systems.

Methods Employed:

Our initial efforts will be directed to making highly specific anti-immunoglobulin. By employing ferritin labeled antibody reagents and electronmicroscopy we hope to gain a better understanding of the distribution of such immunoglobulin receptor molecules.

Major Findings:

Subsequent to the submission of the previous progress report the additional important finding was made that the IgE receptors changed their distribution

on the cell surface when reacted with bivalent anti-IgE antibodies at room temperature or higher (e.g. 37°). This phenomenon appears to be identical to the "capping" observed when immunoglobulin bearing lymphocytes are reacted with anti-immunoglobulin anti-sera. The role of this redistribution in histamine release is uncertain (see below).

Significance to Bio-Medical Research and the Program of the Institute:

It is of considerable importance to study the behavior of antibodies on the surface of cells in order to more completely understand the mechanism of the immune response. It is on the cell surface that most of the critical steps of the immune response transpire and it is here that one must direct one search for the ultimate mechanisms by which antigen-antibody reactions initiate specific cellular responses.

Proposed Course:

New reagents are being prepared from burro and sheep anti-sera so that larger amounts of the requisite hybrid antibodies can be prepared. We will shortly begin a study in which we will attempt to correlate the distribution of surface bound IgE with the course of events during histamine release. (After sensitization of cells with appropriate reaginic antibody there appear to be two distinguishable phases after challenge with antigen or anti-IgE. The first phase is Ca independent, the second phase is Ca dependent, is very rapid and leads to histamine release). It will be most interesting to learn if the "capping" is a necessary condition for histamine release and if so at what stage it occurs.

Honors and Awards: None

Publications:

Metzger, H.: The antigen receptor problem. Ann Rev Biochem 39:889-929, 1970

Serial No. NIAMD-ARB-18C
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Receptors for IgE on Mast Cell Plasma Membranes
Previous Serial No.: None
Prinicpal Investigator: Dr. Henry Metzger

Other Investigators: Dr. Ian Wilson
Dr. Valerie Jones

Cooperating Units: National Institute for Medical Research, Mill Hill London
Clinical Research Center, Northwick Park Hospital, Northwick Park

Man Years:

Total:	1/2
Professional:	1/4
Other:	1/4

Project Description:

Objectives:

IgE is a unique class of immunoglobulin which plays a predominant role in the physiological events associated with a variety of allergic phenomena. The immunoglobulin becomes attached to the surface of basophils and mast cells and upon exposure to the allergenic antigen mediates the release of histamine and other vasoactive amines from the cells to which it is attached. Although there is an increasing amount of information concerning the biochemistry of this reaction almost nothing is known about how the IgE acts as the cell surface transducer. The present project is directed towards increasing our understanding of how the IgE interacts with mast cells.

Methods Employed:

Rats and mice will be immunized according to protocols which are known to yield relatively high levels of reaginic (IgE) antibodies. In vitro and in vivo methods of assaying these antibodies will be employed. Active sera will be absorbed with the cells from two known mouse mastocytomas. If there is a reduction in the reaginic titre after such absorptions will provide preliminary evidence for receptors for IgE on these cells. The absorption studies will then be followed by more direct investigations of IgE binding e.g. by fluorescent antibody techniques and/or by rosette formation between

the IgE coated cells and red cells coated with the antigen to which the IgE is directed. Should these experiments be positive we will then try to isolate mast cell membranes from these cells as a first step to identifying the receptor responsible for IgE binding.

Major Findings: None

Significance to Bio-Medical Research and the Program of the Institute:

It is of considerable importance to study the behavior of antibodies on the surface of cells in order to more completely understand the mechanism of the immune response. It is on the cell surface that most of the critical steps of the immune response transpire and it is here that one must direct one search for the ultimate mechanisms by which antigen-antibody reactions initiate specific cellular responses.

Proposed Course:

See under Methods above.

Honors and Awards: None

Publications: None

Serial No. NIAMD-ARB-19C
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July, 1971 through June 30, 1972

Project Title: Use of Tetranitromethane to Study Antibody Combining Sites

Previous Serial Number: NIAMD-ARB-14c

Principal Investigator: Dr. Henry Metzger

Other Investigators: Dr. Neil Otchin
Mr. John Lee

Cooperating Units: None

Man Years:

Total:
Professional:
Other:

Project Description:

Objectives:

These studies are intended to shed further light on the structure of immunoglobulin combining sites. Tetranitromethane will more or less specifically introduce nitro groups into the 3 position of tyrosyl groups in proteins under mild conditions. Since tyrosyl groups appear to play an important role in many antibody combining sites we wished to see if modification of such tyrosines would effect the combining activity.

Methods Employed:

Tetranitromethane is used to modify myeloma proteins with defineable binding activity. The amount of nitro-tyrosine which is incorporated is assessed by taking advantage of the spectral properties of nitro-tyrosine. Nitration is carried out in the presence and absence of ligand to see a) if the presence of the ligand effects the level of nitration observed and b) to see if the effects of nitration can be modified. Changes in the activity of the proteins are assessed by equilibrium dialysis.

Major Findings:

The findings described previously with regard to the protein IgM_{Wag} were confirmed in repeated experiments. Good quantitative data were obtained which showed that there is a single, hyper-reactive tyrosine in the nitrophenyl binding sites of this protein which when nitrated completely inhibits the binding.

This nitrotyrosine was modified by reduction with dithionite thereby producing 3-aminotyrosine at that position. Because of the unusual pK of this aryl amino group reaction with such reagents as F_2DNB can occur at pH's where ordinary amino groups are unreactive. This permitted us to react the modified tyrosine in IgM_{Wag} with radioactive B_2DNB and to demonstrate that the tyrosine in question was in the Fd region of the molecule.

Significance to Bio-Medical Research and the Program of the Institute:

Nitration of tyrosines is a particularly useful test for probing combining sites for the following reason. The nitrotyrosine can be readily reduced to 3-aminotyrosine (3AT) with dithionite. By virtue of its low pH the aryl amino group of 3AT can be selectively modified with such reagents as radioactive dinitrofluorobenzene, dansylchloride, cross-linking reagents etc. This will provide an opportunity to relatively easily identify the peptides containing the tyrosines in question and to conduct "mapping" experiments with the cross-linking reagents. These studies are part of a series of studies being conducted in this laboratory intended to further our understanding of the structure of immunoglobulin combining sites.

Proposed Course:

This project has been completed and we do not plan to continue this particular approach any further.

Honors and Awards: None

Publications:

Otchin NS, Metzger H: Nitration of a Nitrophenyl-Binding Waldenstrom IgM Macroglobulin. J Biol Chem 246:7051-7057, 1971

Serial No. NIAMD-ARB-20C
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Enrichment of Antigen-Receptor Bearing Immune Cells

Previous Serial Number: 20C

Principal Investigator: Dr. Paul H. Plotz

Other Investigators: Dr. William E. Seaman

Cooperating Units: None

Man Years:

Total:
Professional:
Other:

Project Description:

Objectives:

To obtain enriched population of immune cells so that the antigen receptor, site of initial antigen recognition, may be studied in order to understand how antigens initiate immune responses.

Methods Employed:

Lymphoid cells from immunized mice are exposed to polymer beads with antigen coupled to their surface. Cells are studied before and after exposure to beads to determine whether there is depletion of immune compentence. Attempts are made to remove cells sticking to the beads.

Lymphoid cells from immune mice are exposed to antigen in solution and then exposed to macrophage cultures to determine if immune competent cells are specifically removed.

Major Findings: None

Significance to Bio-Medical Research and the Program of the Institute:

Understanding of foreign antigen recognition and the triggering process in immunity will advance understanding of the origin of the abnormal immune responses seen in patients with autoimmune diseases.

Proposed Course: The studies will continue.

Honors and Awards: None

Publications: None

Serial No. NIAMD-ARB- 21C
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Delayed sensitivity to hepatitis B antigen (HBAg) in patients with hepatitis

Previous Serial Number: None

Principal Investigator: Dr. Paul H. Plotz

Other Investigators: Dr. Saul Agus

Cooperating Units: Digestive & Hereditary Diseases Branch, NIAMD

Man Years:

Total:	1/2
Professional:	1/4
Other:	1/4

Project Description:

Objectives:

To examine the development of delayed sensitivity to the antigen of serum hepatitis in patients with hepatitis in order to discover whether immunity is responsible for the pathogenesis of the disease.

Methods Employed:

Peripheral lymphocytes from patients with acute or healed hepatitis and suitable controls will be cultured in the standard lymphocyte transformation condition. They will be exposed to phytohemagglutinin and HBAg and the cultures examined for incorporation of ^{3}H-thymidine into DNA.

Major Findings: None

Significance to Bio-Medical Research and the Program of the Institute:

The pathogenesis of hepatitis and the reason for the transition from acute to chronic hepatitis may be clarified by studying the development of immunity to the hepatitis virus.

Proposed Course: The studies will continue

Honors and Awards: None

Publications: None

Serial No. NIAMD-ARB-22C
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Cell Mediated Immunity in Myocarditis

Previous Serial Number: None

Principal Investigator: Dr. Kent Salisbury

Other Investigators: Dr. Stephen Guss

Cooperating Units: National Cancer Institute

Man Years:

Total:	3/4
Professional:	1/2
Other:	1/4

Project Description:

Objectives:

Virtually all studies in human autoimmune disease have been concerned with the presence or absence of humoral antibody or antigen-antibody complexes in deciding whether the disease has an immune basis or not. The presence of lymphoid infiltrates in several "immune" disorders raises the important question of the role of cell mediated immunity in addition to humoral immunity. Patients with Systemic lupus erythematosus frequently suffer from congestive heart failure, and yet they infrequently have anti-heart antibody in their serum. Patients with rheumatic heart disease frequently have anti-heart antibodies but less frequently have congestive failure except advanced valvular disease. The question is therefore whether the "heart-sensitized" lymphocyte exists, and whether it plays any role in the pathogenesis of cardiac dysfunction in these and other myocardial diseases, and whether the antibody is in fact a "blocking" antibody, protecting cardiac tissue from cell mediated damage.

Methods Employed:

Rats are immunized with lyophilized whole rabbit heart in Freund's adjuvant either by single or repeated immunization. Serum is collected at regular intervals and examined for anti-heart antibody by immunofluorescence on frozen cardiac tissue, with appropriate controls. Peripheral blood lymphocytes are collected, purified on ficoll-hypaque gradients and then examined for

tritiated thymidine uptake in the presence of soluble cardiac antigens prepared by several methods. The presence or absence of antibody and lymphocyte transformation is then correlated with histologic examination of cardiac tissue for myocarditis. A syngeneic system is used to avoid problems of histo-incompatibility cell transformation (i.e. cultures are performed with rat heart extracts).

Major Findings:

1. The lymphocyte transformation assay does reveal thymidine uptake by peripheral blood lymphocytes from animals immunized with cardiac tissue when exposed to extracts of heart in culture. Control lymphocytes and immune lymphs exposed to other tissue extracts do not transform.

2. This transformation reaction can be demonstrated a full 10 days before antibody can be demonstrated in the serum. Immunofluorescent antibody is produced in high titer 3 weeks after initial immunization.

3. At the time the lymphocyte transformation is demonstrated only minimal myocarditis has been demonstrated in these preliminary experiments.

4. Preliminary examination of two patients with SLE and pancarditis demonstrated low-grade but definite lymphocyte transformation when challenged with soluble cardiac antigen. Patients with SLE and no congestive failure or myocarditis and normal controls demonstrated no transformation to cardiac antigens. The lymphocyte transformation assay therefore, may be a valuable non-invasive method of looking for cell mediated myocarditis in humans.

Significance to Bio-Medical Research and the Program of the Institute:

It is of great importance in the understanding of autoimmune diseases in general and of myocarditis in particular to know the function of cellular immunity. Certainly in cardiac transplantation, but also possibly in disorders such as Dressler's syndrome, or post-pericardotomy syndrome as well as other "idiopathic" myocarditides the small lymphoctye may be the offending agent. If this can be demonstrated then we shall better understand the pathogenesis of the disorders and different therapeutic modalities may be considered on a more rational basis than at present.

Proposed Course:

Further and more detailed correlative studies in animals and humans of lymphocyte function, humoral antibody and clinical and histologic myocarditis are under way. The issue of blocking antibody to cell mediated myocarditis has yet to be evaluated in in vivo and in vitro systems.

Honors and Awards: None

Publications: None

Serial No. NIAMD-ARB-23C
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on a Mouse Myeloma Protein Having Antibody Activity

Previous Serial Number: NIAMD-ARB-13c

Principal Investigator: Dr. Henry Metzger

Other Investigators: Dr. Nortin M. Hadler

Cooperating Units: None

Man Years:

Total:	3/4
Professional:	1/2
Other:	1/4

Project Description:

Objectives:

As outlined previously the availability of homogeneous immunoglobulins with specific binding activity chemically indistinguishable from conventional antibody activity makes it possible to conduct studies which had been essentially impossible to perform on heterogeneous antibodies: structural details of combining sites, role of light and heavy chains in the formation of the combining sites, etc.

Methods Employed:

It was previously shown that the nitrophenyl binding γA myeloma protein from the mouse tumor MOPC 315 could be 'affinity labeled' with meta-nitrobenzene diazonium fluoroborate. A single tyrosine - Tyr_{34} - on the light chain was thereby derivatized. We have attempted to reduce the azo-tyrosine bond in situ with dithionite with the hope that 3 aminotyrosine (3AT) would thereby be generated. Since the aryl amino group of 3AT has an unusually low pK, it should be possible to selectively derivatize it with cross-linking reagents.

Major Findings:

1. The cross-links produced by F_2DNB on native and affinity labeled, dithionite reduced protein 315 have been compared. With the labeled reduced

protein the reaction is more rapid and two types of light chain links are observed: between light chain Tyr_{34} and some other light chain residue and between light chain Tyr_{34} and an unknown residue in the Fd region. In either case, binding activity - which is lost on affinity labeling but regained after dithionite reduction - is lost. With the native protein the reaction with F_2DNB proceeds somewhat more slowly. When the protein is examined after substantial modification has occurred, the only cross-link observed occurs as an intra-heavy chain link.

2. Attempts to isolate peptides containing the cross-links have so far been unsuccessful.

3. Some studies with the same bifunctional reagent have been performed on the phosphorylcholine binding mouse myeloma protein from tumor TEPC 15. This protein appears to be labeled on two different portions of the light chain (see Project Report 16c). The affinity labeled protein is reduced as above with dithionite. Somewhat more than half the activity returns suggesting that some of the binding sites have returned to their native binding capacity. (The association constant for these recovered sites is within experimental error the same as for the unmodified sites). Other sites appear to be completely inactivated. Studies performed in the presence of absence of the ligand phosphorylcholine suggest that that tyrosine, modification of which leads to inactivation is the one which may be preferentially modified.

Significance to Bio-Medical Research and the Program of the Institute:

Our own work and that of others has already shown that much useful data on the structure of antibody combining sites can be obtained by the use of homogeneous mouse myeloma proteins with recognizable binding activity. Affinity labeling studies on both myeloma proteins and conventional antibodies have shown that when diazonium reagents are used, azotyrosine is a frequent product. The techniques developed in the present study should have wide application.

Proposed Course:

Some further work on the nature of the cross-links will be performed. The project will be more or less completed when Dr. Hadler leaves June 30, 1972.

Honors and Awards:

Publications:

1. Hadler NM, Metzger H: Site Directed Cross-Linking: A New Approach to Mapping Antibody Combining Sites. Proc Nat Acad Sci 68:1421-1424, 1971

Serial No. NIAMD-ARB-24C
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on the Combining Sites of Mouse Myeloma Proteins Which Bind Phosphoryl Choline

Previous Serial Number: NIAMD-ARB-16c

Principal Investigator: Dr. Henry Metzger

Other Investigators: Dr. Bruce Chesebro

Cooperating Units: None

Man Years:

Total:	1-3/4
Professional:	1
Other :	3/4

Project Description:

Objectives:

One of the most common activities found among mouse myeloma proteins is the ability to bind phosphoryl choline or its analogues. There already exist 6-8 such proteins which share this property but which are clearly distinguishable on the basis of their binding constant and specificity. These proteins therefore seem ideally suited to study structural functional relationships in immunoglobulin combining sites. Furthermore, since the ligand phosphoryl choline is quite different than the aromatic ligands which have been most frequently studied in immunochemistry some new features of the combining sites of these proteins may become evident.

Methods Employed:

To begin our work it was necessary to purify the proteins and to obtain a suitable labeling reagent. We were able to accomplish both by synthesizing the compound pNO_2 phenyl phosphoryl choline (PNPC) a compound for which no reproducible synthesis had heretofore existed.

Major Findings:

We previously reported that we had successfully affinity labeled several phosphorylcholine binding mouse myeloma proteins with the compound

p-diazoniumphosphorylcholine. The major labeled peptide from one of these labeled proteins (TEPC 15) has now been isolated and characterized. This light chain tryptic peptide has the sequence: Val-His-Tyr*-Leu-Ala-Trp-Tyr-Gln-Lys (The asterisk indicates the residue which was modified). This sequence provides sufficient data to approximately position the peptide in the linear sequence of the TEPC 15 light chain on the basis of its homology with other mouse and human K-type light chains. The most remarkable aspect of this result is that the labeled tyrosine appears to be precisely homologous to the ligh chain tyrosine which became modified when the nitrophenyl binding mouse myeloma protein (MOPC 315) was affinity labeled with m-nitrobenzene diazonium. This was unexpected since these proteins bind such different ligands and since the labeling reagents are so different. That is in the case of the nitrophenyl reagent the reactive diazonium group can be thought of as actually forming part of the determinant whereas with the phosphoryl-choline reagent the diazonium group could be as much as 5-6A away from the main determinant. Based on these findings we concluded (see references below) a) that the combining sites of all immunoglobulins are likely to be in the same topological area of the Bab regions, b) that the modified tyrosine may be particularly reactive for other than steric reasons, and c) that the tyrosine in question is in the combining site and to serve a similar function in antibodies of diverse specificities.

Significance to Bio-Medical Research and the Program of the Institute:

The significance of studying functional myeloma proteins has been discussed in previous Progress Reports. As indicated in the introduction the particular set of proteins should be unusually instructive as far as studying immunoglobulin combining sites.

Proposed Course:

1. In addition to the major labeled peptide referred to above, an additional tyrosine-containing light-chain peptide becomes labeled to a considerable extent. We are attempting to isolate, purify and characterize this labeled peptide.

2. The labeling of the other phosphorylcholine binding myeloma proteins will be further characterized.

Honors and Awards: None

Publications:

1. Chesebro B, Metzger H: Affinity Labeling of a Phosphorylcholine Binding Mouse Myeloma Protein. Biochemistry 11:766-771, 1972

2. Metzger H, Chesebro B, Hadler N, Lee J, Otchin N: Modification of Immunoglobulin Combining Sites. Progress in Immunology 1971, pp. 253-267

Serial No. NIAMD-ARB-25C
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Clinical and Immunological Studies in Sjogren's Syndrome

Previous Serial Number: NIAMD-ARB-5C

Principal Investigator: Dr. Norman Talal

Other Investigators: Dr. Thomas A. Tarpley (D-OMS), Dr. Gerald L. Schall (CC-NM), Dr. Richard Asofsky (I-LMI), Dr. Philip Lightbody (D-DS), Dr. Larry Anderson and Dr. Norman Cummings (D-OMS)

Cooperating Units: National Institute of Dental Research, Dental Services BRanch, Oral Medicine and Surgery Branch; Clinical Center, Department of Nuclear Medicine; National Institute of Allergy and Infectious Diseases, Laboratory of Microbial Immunity

Man Years:

Total:	0
Professional:	0
Other:	0

Project Description:

Due to the departure of the Principal Investigator, this project has been terminated. The projected collaborative clinical study of the efficacy of cyclophosphamide in Sjogren's syndrome was not undertaken because of the imbalance between the relatively benign disease and the relatively toxic therapeutic proposal.

Publications:

1. Waldmann TA, Johnson JS, Talal N: Hypogammaglobulinemia associated with accelerated catabolism of IgG secondary to its interaction with an autoreactive monoclonal IgM. J Clin Invest 50:951-959, 1971

2. Schall GL, Anderson LG, Wolf RO, Herdt JR, Tarpley TM, Cummings NA, Zeigler LS and Talal N: Xerostomia in Sjogren's syndrome. Evaluation by sequential salivary scintigraphy. J A M A 216:2109-2116, 1971

3. Cummings NA, Schall GL, Asofsky R, Anderson LG, Talal N: Sjogren's syndrome - newer aspects of research diagnosis and therapy. Ann Intern Med 75:937-950, 1971

4. Anderson LG, Talal N: The spectrum of benign to malignant lymphoproliferation in Sjogren's syndrome. Clin Exp Immunol 10:199-221, 1972

5. Anderson LG, Cummings NA, Asofsky R, Hylton MB, Tarpley TM, Tomasi TB, Wolf RD, Schall GL, Talal N: Salivary gland immunoglobulins and rheumatoid factor synthesis in Sjogren's syndrome. Natural history and response to treatment. Am J Med (In press)

6. Talal N: Sjogren's syndrome. In Hollander, J. L. (Ed.): Arthritis and Allied Conditions. Philadelphia, Pa., Lea and Febiger, 8th Edition (In press).

Serial No. NIAMD-ARB-26C
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Analysis of the Effect of Diet on the Urinary Constituents of Normal Volunteers

Previous Serial Number: 22C

Principal Investigator: Dr. Peter Goldman

Other Investigators: Dr. Donald S. Young (CC:CP)
Dr. Sheldon Milstien

Cooperating Units: Clinical Center, Clinical Pathology Department

Man Years:

Total:	1-1/2
Professional:	1
Other:	1/2

Project Description:

Objectives:

The object of this study is to determine the effects of diet, particularly a wholly synthetic diet on the chemical constituents of the blood and urine. The major emphasis is to see which ultraviolet-absorbing and carbohydrate-reactive constituents of urine reflect human physiology rather than dietary components.

Methods Employed:

Normal volunteers or patients are given alternate periods of standard Clinical Center diet and the totally synthetic Vivonex diet. Values are determined for constituents of blood and urine normally analyzed by the clinical chemistry laboratory. In addition, the urine is analyzed for carbohydrate, ultraviolet-absorbing and ninhydrin-reactive components. Comparisons are made of the analyses of body fluids while the patients are on the Clinical Center diet and on the synthetic diet.

Guandinosuccinic acid has been measured in the urine of volunteers on these two diets and in two diets which differ in their content of protein.

Major Findings:

On the synthetic diet all normal volunteers showed a decrease in serum urea nitrogen and glucose but a rise in uric acid. The high resolution analysis of the urinary components indicates that by the fifth day of the synthetic diet a new steady state is reached in which a marked simplification of the number of compounds is seen. Nevertheless it would appear from the marked reduction in certain constituents that a greater time on the synthetic diet would yield a greater simplification of the urinary patterns. A striking finding is the rather wide variation in the values of the 14 individuals so far studied on the standardized synthetic diet. The results of the measurements of standard clinical chemical tests on blood and urine have not yet been analyzed, nor have later time periods on the synthetic diet.

Guanidinosuccinic acid (GSA) excretion in the urine is diminished when the volunteer is changed from the conventional to the synthetic diet. The lower level of GSA is achieved within 5 days and then remains constant during the succeeding 5 days on the synthetic diet. This finding indicates that GSA does not arise as the result of the transformation of some dietary constituent e.g., cananavanine. Presumably GSA excretion is related to the nitrogen content of the diet and it is diminished on the synthetic diet which has a lower nitrogen content than the conventional Clinical Center diet. This view is supported by the observation that volunteers on a high protein diet excrete more GSA than these on a low protein diet.

Significance to Bio-Medical Research and the Program of the Institute:

The accurate measurement of compounds in urine and serum that have previously not received attention may offer the opportunity to develop new chemical approaches to the evaluation of human health and disease. Metabolic alterations occurring in certain diseases may be discovered which will lead to a further understanding of the disease process. In addition, by establishing "normal values" for compounds in human body fluids, new diagnostic aids for the early detection of disease may be developed. These normal values or values obtained under standardized conditions may be of value also in providing a basis for evaluating the effect of various drugs on the metabolism of a human subject.

Proposed Course:

It is intended to obtain and evaluate the analytical data from the 14 volunteers from whocm urine and blood samples were taken during the periods of conventional and synthetic diet. The further course of the project will be determined by the results of these findings.

Honors and Awards: None

Publications:

Young DS, Epley JA, Goldman P: Influence of a chemically defined diet on the composition of serum and urine. Clin Chem 17:765-773, 1971

Serial No. NIAMD-ARB-27C
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Metabolic Transformations of Certain Compounds that May be Significant in Human Health and Disease

Previous Serial Number: 21C

Principal Investigator: Dr. Peter Goldman

Other Investigators: Dr. Mark A. Peppercorn
Dr. Sheldon Milstien
Dr. Barry Goldin

Cooperating Units: None

Man Years:

Total:	3-1/4
Professional:	2-1/2
Other:	3/4

Project Description:

Objectives:

To elucidate the origins and normal fate of compounds appearing in human body fluids whose role in human metabolism has not been previously understood.

Methods Employed:

The metabolism of drugs and normal constituents of body fluids have been examined in germfree, gnotobiotic and conventional rats as well as in normal volunteers. The effect of diet on these transformations has been investigated. In addition various bacteria isolated from the gastrointestinal tract of man and experimental animals have been examined for their ability to make the metabolic transformations that have been observed in the human or the experimental animals. The metabolic products have been characterized by the techniques of analytical biochemistry.

Major Findings:

It has been shown that none of 12 bacteria isolated from the human gastrointestinal tract is capable of more than one reaction in the metabolic disposition of caffeic acid which occurs in man and experimental animals. These

results suggested that caffeic acid metabolism is the result of the interactions of different bacteria within the gastrointestinal tract and raise the question of whether the distribution of caffeic acid metabolites as they appear in the urine might be correlated with the nature of the intestinal microflora at the host. Accordingly germfree rats which are incapable of transforming caffeic acid (except in o-methylation) were selectively infected with various strains of bacteria and the capacity of these gnotobiotic rats to transform caffeic acid was investigated. It was found that some categories of these gnotobiotic rats were capable of carrying out reactions of caffeic acid transformation, presumably as the result of the action of the bacteria. However, there was not necessarily any correlation between the metabolic capability of the bacteria as they exist in the gnotobiotic animals and their ability to transform caffeic acid and its metabolites when in the bacterial culture medium.

Azulfidine, a drug used for the treatment of inflammatory disease of the bowel, is another compound in which metabolism by the gastrointestinal organisms appears to be significant. When this drug is fed to conventional rats, none of it is recovered in the urine, feces or cecum, the excreta contain only the products of azo bond reduction, sulfapyridine and 5-aminosalicylate and their metabolites. When the intestinal microflora of conventional rats is decreased by the administration of neomycin, unchanged Azulfidine is recovered in the cecal contents and feces. When an oral dose of Azulfidine is given to germfree rats, recoveries of the drug in urine and feces are over 50%, while none of its metabolites are detected. Gnotobiotic rats (infected with 4 strains of bacteria normally found in the gastrointestinal tracts of rodents) are capable of metabolizing Azulfidine in the manner of conventional rats. These bacteria of the gnotobiotic rats are themselves capable of reducing Azulfidine when they are cultivated on laboratory media in the presence of the drug. Thus it appears that the initiation of Azulfidine metabolism reduction of the azo bond, can be attributed to the intestinal microflora of the host.

Guanidinosuccinic acid (GSA) excretion is increased in patients with the uremic syndrome. Since a specific assay for this compound is not available a soil bacterium has been isolated which can utilize GSA as a sole source of carbon and nitrogen. An enzyme isolated from this organism has proved almost completely specific for the hydrolysis of GSA, the products of this reaction being L-aspartate and urea. They only other compound hydrolyzed by the enzyme is guanidinoglutaric acid and with this substrate the rate of hydrolysis is only about 4% of that with GSA. Furthermore, this other compound has not been found as a constituent of body fluids. This enzyme has been characterized and has been utilized as a specific assay for GSA in studies directed at establishing the physiological parameters that govern the excretion of this compound in both human and experimental animals. The enzyme has been of particular value in relating the configuration of GSA in urine to that of L-aspartate.

Although the origins of GSA have not been determined, several factors governing its metabolism have been elucidated. It has been found that GSA excretion in rats is elevated when the rats are given a diet high in nitrogen. The intestinal microflora may have a role in the degradation of GSA. This is suggested by the observation that germfree rats excrete more GSA than conventional animals and the finding that cultures of several organisms isolated from feces are capable of degrading GSA.

Significance to Bio-Medical Research and the Program of the Institute:

In experimental animals it has been found that the character of the intestinal microflora determine many aspects of the well-being of the host. The findings with Azulfidine are relatively clearcut: namely that the character of the intestinal microflora is apt to determine the therapeutic success with this drug. The findings also raise the question of whether it is Azulfidine or one of its metabolic products formed in the colon which is the active therapeutic agent.

Although caffeic acid is a constituent of our diet, there is no indication that its transformation by the intestinal microflora is of any consequence to the well-being of the host. Nevertheless the finding that there is no correlation between the metabolism of this compound by bacteria of the gnotobiotic rat as they exist in situ or as they behave in culture has implications to those who would relate the incidence of certain disease to the metabolic capacity of the intestinal bacteria.

The increased excretion of GSA in the urine of uremic patients has suggested that this compound is a "uremic toxin." Although this is only a speculation the association of the elevated levels of GSA with the uremic syndrome suggests that an understanding of factors governing the metabolism of this compound may lead to a better understanding of the uremic state.

Proposed Course:

The metabolism and distribution of Azulfidine in humans is now under study to see what role the activity of the intestinal microflora may play in the clinical pharmacology of this drug. Further studies are under way in an attempt to elucidate the metabolic pathway responsible for GSA production and the factors which regulate this pathway.

Publications:

1. Peppercorn MA, Goldman P: Caffeic acid metabolism by bacteria of the human gastrointestinal tract. J Bact 108:996-1000, 1971

2. Peppercorn MA, Goldman P: The role of intestinal bacteria in the metabolism of salicylazosulfapyridine. J Pharm Expt Therapeutics (In press)

3. Peppercorn MA, Goldman P: Caffeic acid metabolism by Gnotobiotic rats and their intestinal bacteria. Proc Nat Acad Sci (In press)

Honors and Awards: None

Serial No. NIAMD-ARB-28C
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Characteristics of the Carbon-Halogen Bond in Compounds of Biological Importance

Previous Serial Number: 23C

Principal Investigator: Dr. Peter Goldman

Other Investigators: Mr. Jay C. Unkeless

Cooperating Units: None

Man Years:

Total:	0
Professional:	0
Other:	0

Project Description:

No further experimental work has been done on this project during this year.

Publications:

1. Unkeless JC, Goldman P: The diastereomers of γ-fluoroglutamate: Complementary structural analogues. Mol Pharmacol 7:293-300, 1971

2. Unkeless JC, Goldman P: Thermodynamic and kinetic aspects of the reaction of γ-fluoroglutamate with D-glutamate cyclase. J Biol Chem 246: 2354-2359, 1971

3. Goldman P: Enzymology of the carbon-halogen bond. In Dagley, S. and Kilgore, W. W. (Eds). Degradation of Synthetic Organic Molecules. Washington, D. C., The National Academy of Science, The National Research Council, 1971, (In press)

4. Goldman P: The use of microorganisms in the study of fluorinated compounds. In R. Peters (Ed.). Carbon-Fluorine Compounds: Chemistry, Biochemistry and Biological Activities. Ciba Foundation Symposium, 1971, (In press)

Serial No. NIAMD-ARB-29C
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Metabolic Actions of Salicylic Acid and Aspirin

Previous Serial Number: 25C

Principal Investigator: Dr. Peter Goldman

Other Investigators: Mr. Jay C. Unkeless

Cooperating Units: None

Man Years:

Total:	0
Professional:	0
Other:	0

This project has been terminated.

Section on Mineral Metabolism

A means has been developed for purifying renal receptors for calcitonin and parathyroid hormone. A series of homogenization and density gradient steps allowed recovery of a fraction of renal membranes enriched with respect to adenyl cyclase, sodium-potassium ATPase and receptors for parathyroid hormone and calcitonin. The receptors were identified by determining binding of radioiodinated hormones to membranes as well as by assays for adenyl cyclase. Preparations of brush borders from renal tubules (kindly provided by Dr. David Neville) are not enriched with respect to hormone-stimulated adenyl cyclase. It is concluded that the plasma membrane fractions purified in our laboratory represent the basal portions of renal tubular cells. Iodinated calcitonin, synthetic of salmon type, binds specifically with high affinity to the membranes. The K_m for binding is approximately 10^{-10} M. It should be possible to develop this system into a highly sensitive radioreceptor assay for calcitonin.
[Drs. Marx, Heath and Aurbach]

Some suggestive evidence was obtained that microtubules of bone cells might be involved in the physiological response to parathyroid hormone. Colchicine injected into rats causes hypocalcemia and prevents the rise in serum calcium following injection of parathyroid hormone into thyroparathyroidectomized rats. The hypocalcemic effect of colchicine appears to be brought about through suppression of bone resorption.
[Drs. Heath and Aurbach]

Receptor for catecholamines in plasma membrane of avian erythrocytes.
A plasma membrane fraction has been prepared from turkey erythrocytes and this fraction contains adenyl cyclase responsive to catecholamines. The receptor for catecholamines in the plasma membrane of the erythrocytes was studied by determining binding of tritiated isoproterenol to the preparation. Catecholamine binds rapidly to the preparation at 37°; binding is essentially complete within 10 to 12 minutes. This binding is specific for catecholamines; tracer hormone is displaced by isoproterenol, epinephrine, norepinephrine, dopamine and dopa, but not by alpha-adrenergic agonists or antagonist. Treatment of the membranes with trypsin allows release of the receptor from the remainder of the membrane fraction. This "solubilized" receptor shows the same high specificity for catecholamines as the plasma membrane preparation itself. Further characterization and purification of the receptor are in progress.
[Drs. Bilezikian and Aurbach]

A series of studies has been carried out utilizing selective venous catheterization and a radioimmunoassay for parathyroid hormone for localization of parathyroid adenomas. A high degree of success has been obtained in localizing these tumors preoperatively. The catheter is passed through the femoral and thence through the major veins to reach the neck where selective samples are taken from each inferior thyroid and superior thyroid vein. Radioimmunoassays show that veins draining parathyroid adenomas frequently contain concentrations of hormone 10 to 20-fold or more greater than the concentration in the peripheral plasma. In cases with single adenomas, this concentration ratio is greatest selectively in one vein draining the adenoma. The contralateral vein shows concentrations equal to peripheral results. Patients with hyperplasia of all four glands show concentrations of hormone in the thyroid veins on each side greater than concentrations in peripheral plasma. Usually in these cases the concentration ratio is not as high as for single adenomas. This technique utilizing specific radioimmunoassays and selective catheterization should be applicable also to determining the source of hormone in ectopic hyperparathyroidism produced by non-parathyroid cancers.
[Drs. Marx, Bilezikian and Aurbach, NIAMD, Drs. Pearson and Kumpe, Radiology Department, C. C., and Drs. Powell and Potts, Massachusetts General Hospital]

Specific receptors for glucose in the glucose transport system of the isolated renal tubule have been identified. A procedure was developed utilizing radioiodinated phloridzin to identify transport receptors for glucose. Two receptors for phloridzin were found in isolated renal tubules from the renal cortex. One type of receptor is a high affinity system with a K_m of approximately 5×10^{-7} M that is affected by phenols as well as sugars at very high concentrations. The low affinity-high K_m system is stable to boiling. It was also proved in contradistinction to results of other workers, that phloridzin indeed is transported by renal tubular cells.
[Drs. Heath and Aurbach]

Further support has been gained for the thesis that the mechanism of action of parathyroid hormone is mediated through activation of adenyl cyclase in bone and kidney and consequent rise in tissue concentration of 3',5'-AMP. Dibutyryl-cyclic 3',5'-AMP added to fetal bones in vitro produces biological effects qualitatively similar to those effected by parathyroid hormone. It was found that the action of dibutyryl-3',5'-AMP can be attributed to induction of a rise in endogenous tissue concentration of 3',5'-AMP itself. This increase in tissue concentration of the cyclic nucleotide is brought about by inhibiting cyclic nucleotide phosphodiesterase in the cell. Studies with tracer amounts of dibutyryl-3',5'-AMP proved that the concentration of the latter reached sufficient concentrations within the cell to effectively inhibit endogenous phosphodiesterase. Heretofore it had been thought that dibutyryl-3',5'-AMP acted with cells as an analog of 3',5'-AMP by activating the same intracellular systems affected by the natural cyclic nucleotide.
[Drs. Heersche and Aurbach, NIAMD]

Section on Physiology and Clinical Nutrition

At the beginning of FY 1972, Mr. Joseph Muenzer left SPCN to enter graduate and medical training at Case Western Reserve Univesity, and was replaced by Mr. Theodore Watkins. At mid-year, Dr. Sidney Wolfe terminated his full-time association with the laboratory, and for the latter half of the year has been able to devote only a small part of his time to research. He will separate from NIH entirely when FY 1973 begins. His contributions have been valuable, and he will be missed. Dr. Droller will also complete his two-year associateship at the end of FY 1972.

Investigations into the physiology and metabolism of human sweat glands *in vivo* continue to be the major concern of the metabolic chamber group. A series of experiments with ^{14}C urea in normal volunteers has been completed; it shows clearly that urea, originally derived from the extracellular pool, lingers in some delay pool before being excreted in sweat. Immediately after a dose of tracer urea is given, sweat urea is about 2/3 as radioactive as blood urea, whereas 3 or 4 days later, it may have twice or more the specific activity of blood urea. The localization of this delay pool is still a matter of conjecture, although it seems certain that it must lie somewhere in the skin. Attempts to leach out the trapped urea by the application of water to the skin surface have not yielded convincing success. The possibility that sweat urea concentrations exceed those of blood because of synthesis of urea in the sweat gland is ruled out by this study, and the results make it highly improbable that the excess urea in sweat is related to passive water reabsorption in the sweat duct, as was previously thought. In another study, the effect of three days' starvation on sweat gland function was investigated, using normal volunteers. Although such fasting largely exhausts the human body's supply of stored carbohydrate and causes almost all vital organs and tissues to convert their energy metabolism to a dependence on the oxidation of fatty acids and ketones, this appears not to apply to sweat glands. Their output of lactate fell significantly, but the amount of lactate excreted in sweat remained high. We have calculated the quantitative impact of heavy sweating on the economy of carbohydrate in a fasting man, and have concluded that the demands of the sweat glands for glucose could increase the requirement for gluconeogenesis from catabolized protein by nearly 50%. The effect of three days' fasting on sweat gland glycogen content is now under investigation.

[Drs. Robert S. Gordon, Jr. and Ronald H. Thompson, and Mr. Del Thrasher]

The physiology of human blood platelets has been the other prime focus of research in the section during FY 1972. Washed platelets, resuspended in artificial media, undergo chemical changes on the application of thrombin, and release intracellular nucleotides and calcium into the surrounding fluid. At the same time, they tend to aggregate if this phase of the reaction is not prevented by EDTA or other anti-calcium reagents. Platelets undergoing the release reaction without aggregating have been studied by electron microscopy under a wide variety of chemical circumstances. Three major changes appear to correlate with the degree of release: disappearance of dark alpha granules from the cytoplasm, the formation of a system of intracytoplasmic channels and vacuoles, and the appearance within these spaces of an insoluble substance having the appearance of fibrin. Temperature changes or chemical agents which inhibit these ultrastructural changes, and materials which stimulate release cause the morphologic alterations to develop and progress. It seems highly probable that the alpha granules normally contain stored calcium and nucleotides, and that the formation of the network of channels is intimately related to the process whereby the contents of these granules are transported to the exterior of the platelet.
[Dr. Droller, with EM facilities provided by Dr. N. Feder, LEP]

Biochemical studies of platelet function have centered around the role of cyclic AMP in the mediation of the effects of thrombin and other agents which cause release and aggregation to occur. Previous work elsewhere had indicated that thrombin caused platelet cAMP levels to decrease, an unusual finding in a world where hormones or other humoral agents seem always to act by increasing intracellular cAMP in the target cell. However, using carefully washed platelets and purified human thrombin in small (physiologic) dosages, it has been possible to show an increase in platelet cAMP. It is not clear, though, that this increase in cAMP is in fact the mediator for the release reaction. Firstly, the release reaction is extremely rapid, and is complete at a time when cAMP levels are just beginning to increase. Secondly, either theophylline or PGE_1 will augment the effect of thrombin on platelet cAMP content, but will partially inhibit the release reaction. These studies indicate that the earlier simplistic, though paradoxical, view that thrombin decreased platelet cAMP levels and thus cause them to release and aggregate, and that theophylline and PGE_1 antagonized thrombin by increasing cAMP concentrations, appears to require modification.
[Drs. Droller and Wolfe]

It is of interest that platelets suddenly increase their oxygen consumption when thrombin is applied. Using a polarographic method of great sensitivity, this phenomenon and the effect of a variety of inhibitors on it have been studied. Poisons which inhibit mitochondrial respiration (e.g. cyanide) act much more strongly on basal oxygen consumption than they do on the thrombin-induced burst, while specific inhibitors of platelet function (e.g., PGE_1) show the opposite specificity.
[Drs. S. Wolfe and E. Weinbach (NIAID), and Mr. Joe Muenzer and Mr. Ted Watkins]

Two other projects are now in initial stages, but have not yielded enough results to report here. It appears that exposure to thrombin stimulates local production of prostaglandins by platelets, so that some effects of thrombin may in fact be mediated by these peculiar fatty acids. This phenomenon is to be studied in the next few months, using a radioimmunoassay for prostaglandins. It has long been known that the removal of one kidney from any animal will cause hypertrophy of the remaining organ, but the substances that stimulate this growth have not been identified. Using the methods that have been set up for the platelet studies, it should be possible to determine if changes in adenyl cyclase activity, cAMP concentrations, or prostaglandin levels in kidney tissue are associated with the process of hypertrophy.
[Dr. Droller]

Phoenix Clinical Research Section, Phoenix, Arizona

The major interest of this section has been to determine the causes of lithogenic bile in American Indian women with cholesterol gallstones. Lithogenic bile contains an excess of cholesterol relative to the solubilizing lipids -- bile acids and phospholipids, and because cholesterol exists in a supersaturated state, it tends to crystallize out of solution to form cholesterol crystals and gallstones. Lithogenic bile could be the result of excess hepatic secretion of cholesterol or reduced secretion of bile acids and/or phospholipids. American Indian women, who have the highest prevalence of cholesterol stones of any group in the world, secrete lithogenic bile, and it was our purpose to discover the alterations in lipid metabolism responsible for this abnormal bile. The primary method used in these studies was an intubation technique for estimating the hourly secretion rates of biliary lipids - cholesterol, bile acids, and phospholipids - in Indian women with gallstones. The method required intubation of patients with a 3-lumen tube, and secretion rates were measured by marker dilution techniques. For control subjects, we studied two groups without gallstones -- Indian men and white women. We found that Indian women with gallstones had two abnormalities, as compared to controls. First, they secreted only about half as much bile acids as the two control groups; and second, they put out more biliary cholesterol. Phospholipid secretion was the same for all three groups. Therefore, we concluded that American Indian women have a dual defect in bile lipids: bile acid secretion is reduced and cholesterol output is enhanced. (Drs. Scott Grundy, Allan Metzger, Ronald Adler and Eunice Flock).

In the attempt to determine the causes of these alterations in hepatic secretion of cholesterol and bile acids, cholesterol balance studies were carried out in Indian women with gallstones and in the two control groups without stones--Indian men and white women. Measurements of fecal excretions of cholesterol and bile acids were made. Since the patients were fed low cholesterol diets, the excretion of total steroids (bile acids + cholesterol) should equal the synthesis of cholesterol, and excretion of bile acids should approximate their rate of synthesis. This assumption depends, of course, on the existence of a steady state. It was found that Indian women with gallstones excreted comparable amounts of bile acids as control subjects. Therefore, they did not have an absolute block in bile acid synthesis to account for the low hepatic secretion of

bile acids. On the other hand, they did not have a severe defect in bile acid reabsorption that could explain this reduced secretion; for it has been shown that Caucasians can rapidly replenish bile acid pools and maintain normal secretion rates in the presence of sizeable intestinal losses. Therefore, we conclude that homeostatic regulation of bile acid synthesis is abnormal in Indian women. The defect is in the regulation of bile acid synthesis and not in absolute synthesis rates. Since cholesterol is inadequately converted into bile acids, the hepatic secretion of cholesterol is enhanced. However, the secretion of cholesterol in bile of Indian women with gallstones is further enhanced by an increased synthesis of cholesterol. This increased synthesis in Indian patients, as compared to Caucasian controls, was also demonstrated by the sterol balance method. Whether this increased synthesis is due to obesity, racial differences, or decreased bile acid pools has not been determined, but this question is the subject of present study. (Drs. Ronald Adler, Scott Grundy, Malcolm Tyor, and Allan Metzger).

Finally, it was shown that the lithogenicity of bile in Indian women was periodically increased by a diurnal variation. During the night, the pool of bile acid passes through the liver and into the gallbladder. Since this sequestration of bile acids in the gallbladder occurs during the first part of the night, hepatic bile is relatively deficient in bile acids later in the night and in the early morning hours. However, it appears that secretion of cholesterol and phospholipid continues, and hence, the lithogenicity of hepatic bile increases. Although diurnal variation in bile lipid composition occurs in all people, the abnormality produced in Indian women is accentuated. Therefore, if complete mixing of bile does not occur in the gallbladder, there will be regions of high lithogenicity. These regions might serve as areas for the formation of cholesterol crystals, which could in turn act as a nidus for gallstone formation. (Drs. Allan Metzger, Scott Grundy, Ronald Adler and Eunice Flock).

Serial No. NIAMD-MDB-1 (c)
1. Metabolic Diseases Branch
2. Section on Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies in Bone Metabolism

Previous Serial Number: Same

Principal Investigator: Dr. G. D. Aurbach

Other Investigators: Drs. Stephen Marx, John Bilezikian, James Phang and G. Donald Whedon

Cooperating Units: Dr. Mones Berman, Mathematical Research Branch, NIAMD
Metabolism Branch, NCI

Man Years:

Total:	2.25
Professional:	.25
Other:	2.00

Project Description:

Objectives: 1) To investigate the factors influencing mineral storage and loss in demineralizing bone diseases. 2) To investigate the response of the parathyroid gland to rapid changes in blood calcium. 3) To study the influence of phosphate on resorption of bone mineral.

Methods Employed: 1) Metabolic balance studies in patients with disorders of calcium metabolism, noting the effects on nitrogen, calcium, phosphate and magnesium balances of various dietary intakes of calcium, phosphate and magnesium. 2) Estimation of pool sizes, turnover rates, bone deposition rates, and absorption rates of calcium in patients by the oral and intravenous administration of tracer doses of ^{47}Ca.

Major Findings: Same as those reported 1968-'69, 1969-'70 and 1970-'71.

Honors and Awards: None

Publications:

1. Whedon, G. D., and Lutwak, L.: Metabolic studies of the gemini 7-14 day orbital spaceflight. In Murray, R. H., and McCally, M. (Eds.): Proceedings of Hypogravic and Hypodynamic Environments Conference, 1969. NASA Sp. Report 269, 1971, pp. 51-65.

Serial No. NIAMD-MDB-2 (c)
1. Metabolic Diseases Branch
2. Section on Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Study of Parathyroid Hormone: Physiological Regulation of Secretion; Physical, Chemical, and Immunochemical Properties; Biochemistry; and Structure, and Mechanism of Action.

Previous Serial Number: Same

Principal Investigator: Dr. G. D. Aurbach

Other Investigators: Drs. Stephen Marx, John Bilezikian, David Heath J. T. Potts, Jr., G. W. Tregear, H. Niall, G. P. Mayer, D. Kronfeld, and C. Ramberg.

Cooperating Units: University of Pennsylvania School of Veterinary Medicine and Endocrine Unit, Mass. Gen. Hospital.

Man Years:

Total:	3.00
Professional:	.75
Other:	2.25

Project Description:

Objectives: To prepare purified parathyroid hormone in pilot-plant quantities; to study chemical properties and structure of the hormone; to relate chemical properties and structure to biological activity and to study the mechanism of action of the hormone; to develop a clinically useful test for circulating parathyroid hormone; to study the physiological regulation of secretion of the hormone.

Methods Employed: Pure parathyroid hormone is prepared from phenol extracts of bovine parathyroid glands by solvent fractionation, gel filtration and chromatography on carboxymethylcellulose. Purity is assessed by disc gel electrophoresis, ultracentrifugation, immunodiffusion and immunoelectrophoresis, and recovery of the amino- and carboxyl-terminal amino acids. The hormone is assayed in vivo in rats, in vitro by measuring hormonal activation of adenyl cyclase, or by a highly sensitive radioimmunoassay. Specific chemical and enzymatic methods are used to cleave the

hormone to smaller polypeptide fragments. The polypeptide derivatives are sequentially degraded with exopeptidases and the Edman reaction. Adenyl cyclase is measured by determining conversion of $-^{32}$P-labeled ATP to cyclic 3',5'-AMP. Endogenous concentration of cyclic 3',5'-AMP is determined by 1) converting the cyclic nucleotide to ATP and measuring the latter with a ^{32}P-ATP exchange reaction or 22) radioimmunoassay.

Major Findings: Mechanism of Action.- Our earlier studies showwed that the parathyroid status of animals is an important factor regulating excretion of cyclic AMP in the urine. This finding led to development of the thesis that cyclic AMP is involved in the mediation of parathyroid hormone action. Subsequent work showed that parathyroid hormone in vitro activates adenyl cyclase in the kidney as well as in bone, the two organs known to be physiological receptors for action of the hormone. Adenyl cyclase in the kidney is found in two different anatomic zones; adenyl cyclase in the cortex responds specifically to parathyroid hormone whereas that in the medulla responds to vasopressin. Further studies show that virtually all the adenyl cyclase detectable in kidney tissue is found within the renal tubules. This work establishes that the receptor for parathyroid hormone in kidney resides within the renal cortical tubule as had been suspected but not proven by indirect in vivo physiological experiments.

Addition of parathyroid hormone to fetal rat calvaria incubated in vitro causes a 10-fold increase in the intracellular concentration of cyclic AMP within five minutes. Physiological changes in calcium release begin to occur 1 to 2 hours after the rise in intracellular 3',5'-AMP. Other studies show that dibutyryl-3',5'-AMP (DBC) added in vitro mimics the physiological effects of parathyroid hormone. We have now elucidated the mechanism through which the effects of DBC are produced. After adding dibutyryl-3',5'-AMP in vitro there was a rapid increase in concentration of endogenous 3',5'-AMP. This rise in tissue concentration of 3',5'-AMP can be accounted for by inhibition of DBC of cyclic nucleotide phosphodiesterase within the cell.

A protein kinase from bovine renal cortex has been identified and purified 10-fold. This enzyme catalyzes the ATP-dependent phosphorylation of arginine-rich histone and is activated by the addition of 3',5'-cyclic AMP. The K_m for 3',5'-AMP in this reaction is approximately 10^{-8}M. The reaction requires magnesium, although a slow rate of reaction can be obtained with manganese. Recent studies indicate that it may be possible to isolate the enzyme and this should allow ultimately the identification of the physiological substrate phosphorylated in the kidney and bone in response to parathyroid hormone.

Chemistry of Parathyroid Hormone.- Application of the "sequenator" method to analysis of parathyroid hormone has allowed deduction of the complete amino acid sequence of bovine parathyroid hormone. The molecule is an 84-amino-acid polypeptide with alanine as the amino-terminal residue and glutamine at the C-terminus. There are two phenylalanines (residues 7 and 34), two methionines (residues 8 and 18), one tryptophan (23) and one tyrosine (43). Previous work from this laboratory showed that the biologically important region of the molecule was located at the amino terminus within a polypeptide smaller than that demarcated by the first aspartate residue. Deduction of the sequence of the molecule allowed chemical synthesis of an amino-terminal biologically active fragment. The tetratriacontapeptide representing residues 1-34 in the hormonal molecule was synthesized by a solid-phase method.

After deblocking and purification the synthetic peptide was tested for biological and immunological activity. The product showed qualitatively all the biological properties, in vivo as well as in vitro, of the native molecule. Thus, the protean effects of the hormone on both renal and skeletal tissue can be accounted for by the same limited region of the molecule. It is also implied that the receptor sites for the hormone must be similar in bone and kidney.

Human Parathyroid Hormone.- Parathyroid hormone was purified from human parathyroid adenomas. Earlier studies proved that the human hormone is different immunologically from the bovine hormone. Amino acid analyses of the hormone purified from each source indicate eight or nine amino acid substitutions in the human hormone. These changes are predominantly in the neutral amino acids. It is probable that any major substitutions occur outside the amino terminal region important for biological activity. Substitutions beyond this region undoubtedly account for the immunological distinctions between the hormones of different species.

Efforts are continuing to collect large amounts of human parathyroid tissue from which further quantities of human hormone can be purified. With the highly sophisticated techniques now available for sequence analysis, it is projected that if as much as 1 to 2 mg of human parathyroid hormone can be isolated in pure form it will be possible to determine its amino acid sequence. This would be a major accomplishment in that it would allow synthesis of human parathyroid hormone which would then become a standard for radioimmunoassays and other potential wide application.

A series of studies has been carried out utilizing selective venous catheterization and a radioimmunoassay for parathyroid hormone for localization of parathyroid adenomas. A high degree of success has been obtained in localizing these tumors preoperatively. The catheter is passed through the femoral and thence through the major veins to reach the neck where selective samples are taken from each inferior thyroid and superior thyroid vein. Radioimmunoassays show that veins draining parathyroid adenomas frequently contain concentrations of hormone 10 to 20-fold or more greater than the concentration in the peripheral plasma. In cases with single adenomas, this concentration ratio is greatest selectively in one vein draining the adenoma. The contralateral vein shows concentrations equal to peripheral results. Patients with hyperplasia of all four glands show concentrations of hormone in the thyroid veins on each side greater than concentrations in peripheral plasma. Usually in these cases the concentration ratio is not as high as for single adenomas. This technique utilizing specific radioimmunoassays and selective catheterization should be applicable also to determining the source of hormone in ectopic hyperparathyroidism produced by non-parathyroid cancers.

Significance to Biomedical Research and Program of the Institute: Same as for 1968-'69, 1969'70 and 1970-'71.

Proposed Course of Project: Same as for 1968-'69, 1969-'70 and 1970-'71.

Honors and Awards: None

Publications:

1. Aurbach, G. D., and Desbuquois, B.: Radioimmunoassay of parathyroid hormone. In Sunderman, F. W., and Sunderman, F. W., Jr. (Eds.): Laboratory Diagnosis of Endocrine Diseases. St. Louis, Mo., Warren H. Green, Inc., 1971, pp. 297-301.

2. Aurbach, G. D., Keutmann, H. T., Niall, H. D., Tregear, G. W., O'Riordan, J. L. H., Marcus, R., Marx, S. J., and Potts, J. T., Jr.: Structure, synthesis and mechanism of action of parathyroid hormone. Recent Prog. Hormone Res. 28: 353-398, 1972.

3. Aurbach, G. D., Marcus, R., Heersche, J., Marx, S., Niall, H., Tregear, G. W., Keutmann, H. T., and Potts, J. T., Jr.: Hormones and other factors regulating calcium metabolism. Ann. N. Y. Acad. Sci. 185: 386-394, 1971.

4. Aurbach, G. D., Marcus, R., Heersche, J. N. M., Winickoff, R. N., and Marx, S. J.: Cyclic nucleotides in the action of native and synthetic parathyroid and calcitonin peptides. In Talmage, R. V., and Munson, P. L., (Eds.): Calcium, Parathyroid Hormone and The Calcitonins. Amsterdam, Excerpta Medica, 1972, pp. 502-510.

5. Desbuquois, B., and Aurbach, G. D.: Use of polyethylene glycol to separate free and antibody-bound peptide hormones in radioimmunoassay. J. Clin. Endocr. 33: 732-738, 1971.

6. Desbuquois, B., and Aurbach, G. D.: Distribution of free and antibody-bound peptide hormones in two-phase aqueous polymer systems. Biochem. J. 126: 717-726, 1972.

7. Heersche, J. N. M., Fedak, S. A., and Aurbach, G. D.: The mode of action of dibutyryl adenosine 3',5'-monophosphate on bone tissue in vitro. J. Biol. Chem. 246: 6770-6775, 1971.

8. Heersche, J. N. M., and Aurbach, G. D.: Cyclic AMP response in bone after prolonged exposure to parathyroid hormone. In Talmage, R. V., and Munson, P. L. (Eds.): Calcium, Parathyroid Hormone and The Calcitonins. Amsterdam, Excerpta Medica, 1972, pp. 511-512.

9. Keutmann, H. T., Aurbach, G. D., Dawson, B. F., Niall, H. D., Deftos, L. J., and Potts, J. T., Jr.: Isolation and characterization of the bovine parathyroid isohormones. Biochemistry 10: 2779-2787, 1971.

10. Marcus, R., and Aurbach, G. D.: Adenyl cyclase from renal cortex. Biochim. Biophys. Acta 242: 410-421, 1971.

11. Potts, J. T., Jr., Keutmann, H. T., Niall, H. D., Habener, J. F., Tregear, G. W., Deftos, L. J., O'Riordan, J. L. H., and Aurbach, G. D.: Chemistry of the parathyroid hormones: clinical and physiological implications. In Talmage, R. V., and Munson, P. L. (Eds.): Calcium, Parathyroid Hormone and The Calcitonins. Amsterdam, Excerpta Medica, 1972, pp. 159-172.

12. Potts, J. T., Jr., Niall, H. D., Keutmann, H. T., Tregear, G. W., Sauer, R., Hogan, M. L., Dawson, B., and Aurbach, G. D.: Chemistry of parathyroid hormone. In Nichols, G., and Wasserman, R. H. (Eds.): Cellular Mechanisms for Calcium Transfer and Homeostasis. New York, Academic Press, Inc., 1971, pp. 393-401.

13. O'Riordan, J. L. H., Potts, J. T., Jr., and Aurbach, G. D.: Isolation of human parathyroid hormone. Endocrinology 89: 234-239, 1971.

14. Heath, D.A., Palmer, J. S., and Aurbach, G. D.: Hypocalcemic action of colchicine. Endocrinology 1589-1593, 1972.

15. Keutmann, H. T., Dawson, B. F., Aurbach, G. D., and Potts, J. T., Jr.: A biologically active amino-terminal fragment of bovine parathyroid hormone prepared by dilute acid hydrolysis. Biochemistry 11: 1973-1979, 1972.

Serial No. NIAMD-MDB-3 (c)
1. Metabolic Diseases Branch
2. Section on Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on the Chemical Nature and Mode of Action of Thyrocalcitonin.

Previous Serial Number: Same

Principal Investigator: Dr. G. D. Aurbach

Other Investigators: Drs. Stephen Marx, David Heath, James Gavin

Cooperating Units: Clinical Endocrinology Branch, NIAMD
Department of Endocrinology, Mass. Gen. Hospital

Man Years:
Total: 1.25
Professional: .75
Other: .50

Project Description:

Objectives: To study the interaction of calcitonin with its specific receptor target organs.

Methods Employed: The interaction of calcitonin with its receptors has been evaluated by determining adenylate cyclase as well as binding of radioiodinated calcitonin to membranes isolated from renal and skeletal tissue.

Major Findings: A purification procedure for kidney membranes has been developed. It has been shown that the calcitonin receptor from the kidney is concentrated at the corticomedullary junction. The membranes which bind calcitonin and contain calcitonin-sensitive adenylate cyclase show low specific activities for renal brush border enzymes, indicating that calcitonin receptors are localized at an anti-luminal aspect of renal cells or at a site that does not contain brush borders. Utilizing a series of calcitonin analogs, dose response curves were compared for receptors from kidney and bone. The dose response curves in the two tissues are virtually superimposable, suggesting that the calcitonin receptor in the two organs has a similar molecular structure.

Significance to Biomedical Research and Program of the Institute: The current investigations should provide further insight into the structure function relationship in calcitonin. Calcitonin is a small polypeptide hormone and therefore lends itself well to studies using synthetic peptide fragments. The system is also useful characterizing hormone receptors present in kidney and bone.

Proposed Course of Project: Further studies are in progress to characterize further the interaction of calcitonin with its tissue receptors. It also will be of interest to solubilize calcitonin receptors and further characterize them.

Honors and Awards: None

Publications: None

Serial No. NIAMD-MDB-4(c)
1. Metabolic Diseases Branch
2. Section on Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 20, 1972

Project Title: Studies on Pseudohypoparathyroidism and Related Disorders

Previous Serial Number: Same

Principal Investigator: Dr. G. D. Aurbach

Other Investigators: Drs. Stephen Marx, John Bilezikian, Thomas P. Nigra and Ervin H. Epstein

Cooperating Unit: National Cancer Institute

Man Years:

Total:	1.75
Professional:	.75
Other:	1.00

Project Description:

In 1942 Albright and his associates described the features of a new clinical syndrome "pseudohypoparathyroidism." Patients with this disorder differ from those with idiopathic hypoparathyroidism in that they have characteristic constitutional features and they do not respond to exogenous parathyroid extract. Albright proposed that this disorder was attributable to lack of end organ sensitivity to normally-secreted endogenous parathyroid hormone. Our findings that parathyroid hormone action is likely mediated through activation of adenyl cyclase, led us to test cases of pseudohypoparathyroidism by giving intravenous parathyroid hormone and measuring cyclic 3',5'-AMP in the urine. Pseudopseudohypoparathyroidism is a variant of the disorder and may occur in the same families with pseudohypoparathyroidism. Pseudopseudohypoparathyroidism is characterized by similar constitutional features but no clinical evidence of hypoparathyroidism. Several other syndromes including Gardner's syndrome, basal cell nevus syndrome, syndrome of calcification of basal ganglia, and vitamin D-resistant osteomalacia have also been reported in the past as resistant to the phosphaturic action of parathyroid hormone. Current studies have been extended to include testing patients in these categories as well.

Methods: Parathyroid hormone purified in our laboratories was sterile-filtered for clinical use through courtesy of the N.I.H. Pharmacy. Radio-immunoassay of parathyroid hormone was carried out as described previously. Measurement of 3',5'-AMP was performed according to the procedure devised in this laboratory.

Major Findings: Approximately 70 cases of this disorder have been described in the literature; we have examined 18 of these to date. In normal subjects, parathyroid hormone causes a 30- to 60-fold rise in urinary excretion of cyclic AMP within 30 minutes or less after intravenous administration of the hormone. A similar response has been observed in idiopathic, hypoparathyroid, surgical hypoparathyroid, and pseudopseudohypo-parathyroid subjects (the latter category show the constitutional changes but do not have laboratory evidence of hypoparathyroidism). Parathyroid hormone caused little or no increase in excretion of cyclic AMP in patients with pseudohypoparathyroidism. In one case, studied through the cooperation of a clinical investigator at another hospital, there was a moderate rise in excretion of cyclic AMP; however, the diagnosis in this instance may be incorrect, and as yet we have not had the opportunity of seeing the patient at the Clinical Center. Patients with this disorder who fail to respond to parathyroid hormone showed a similarly defective response after prolonged calcium infusion. Parathyroid hormone was detectable in the plasma of pseudohypoparathyroid subjects before infusion of calcium but not after. This experiment tended to rule out the possibility that an abnormal endogenously secreted hormone immunologically reactive but biologically inert interfered with the action of exogenous parathyroid hormone.

The best evidence to date is that pseudohypoparathyroidism represents a genetic defect inherited as a sex-linked, dominant trait. There have been no documented cases of direct male-to-male transmission of the disease and the sex incidence is approximately 2:1 female-to-male. Several pedigrees have been described wherein pseudophyoparathyroid subjects were progeny of pseudopseudohypoparathyroid parents or vice versa. One family studied at the N.I.H. included a mother with pseudopseudohypoparathyroidism and her daughter with pseudohypoparathyroidism. The daughter failed to respond to exogenous hormone but the mother responded normally. We have also observed that, although the response to pseudopseudohypoparathyroidism is normal to exogenous hormone, there is a significantly increased rare of baseline excretion of cyclic AMP in this group. This observation may be of importance in further delineation of the precise genetic mechanism involved in trans-mission of the disorder. It appears likely that the cause of the disorder may be the existence of genetically deficient or defective adenyl cyclase in the bone and kidney of these subjects.

There is an association of hypothyroidism with pseudohypoparathyroidism and in a few such cases the hypothyroidism has been attributed to selective deficiency of thyrotropin. It seems possible that hypothyroidism in pseudohypoparathyroidism might reflect an abnormal receptor adenyl cyclase complex in the central nervous system or anterior pituitary analogous to the defect in the receptor for parathyroid hormone in kidney and bone. Three patients with coexistent hypothyroidism and pseudohypoparathyroidism were given thyrotropin-releasing hormone as part of a study designed to localize the site of the thyroid defect. One case showed an abnormally low basal concentration of thyrotropin in plasma, but gave a normal response to thyrotropin-releasing hormone. In the two other cases, a mother and daughter, there were high basal concentrations of thyrotropin in plasma. The mother was tested with TRH and showed the exaggerated response to TSH secretion characteristic of primary hypothyroidism. The defect in the first case appears to be at the level of the hypothalamus or higher. The defect in the second two cases may represent an abnormality of the thyroid receptor for TSH itself.

Patients with Cushing's syndrome, basal cell nevous syndrome, syndrome of calcification of the basal ganglia, and vitamin D-resistant osteomalacia showed normal responses to parathyroid hormone as determined by urinary excretion of 3',5'-AMP. It was concluded that the classical phosphaturic response to parathyroid hormone is neither precise nor sensitive enough to discriminate normal subjects from those refractory to parathyroid hormone.

Kidney tissue was obtained from a patient with pseudohypoparathyroidism who died suddenly with complications of a massive pulmonary embolus. It was demonstrated in vitro that the membrane fraction of this kidney contained adenylate cyclase activity which was stimulatable by parathyroid hormone. Thus, it was concluded that pseudohypoparathyroidism did not represent a total lack of the renal parathyroid hormone-sensitive adenylate cyclase system.

Significance to Biomedical Research and Program of the Institute: Same as reported for 1968-69, 1969-'70 and 1970-'71.

Proposed Course of Project: Same as reported for 1968-'69, 1969-'70, and 1970-'71.

Honors and Awards: None

Publications:

1. Aurbach, G. D.: Genetic disorders involving parathyroid hormone and calcitonin. In Bergsma, D. (Ed.): Birth Defects: Original Article Series. New York, The National Foundation-March of Dimes, 1971, vol. VII, pp. 48-54.

2. Marcus, R., Wilber, J. F., and Aurbach, G. D.: Parathyroid hormone-sensitive adenyl cyclase from the renal cortex of a patient with pseudohypoparathyroidism. J. Clin. Endocr. 33: 537-541, 1971.

3. Marx, S. J., Hershman, J. M., and Aurbach, G. D.: Thyroid dysfunction in pseudohypoparathyroidism. J. Clin. Endocr. 33: 822-828, 1971.

4. Moskowitz, M. A., Winickoff, R. N., and Heinz, E. R.: Familial calcification of the basal ganglions. New Eng. J. Med. 285: 72-77, 1971.

Serial No. NIAMD-MDB- 5(c)
1. Metabolic Diseases Branch
2. Section on Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Study of Hormone-Mediated Solute Transport: Interaction of Hormones with Cell Receptors; Interrelationship between Biogeneration of Cyclic AMP and Active or Facilitated Transport.

Previous Serial Number: None

Principal Investigator: Dr. G. D. Aurbach

Other Investigators: Drs. Stephen Marx, John Bilezikian and David Heath

Cooperating Units: Digestive and Hereditary Diseases Branch, NIAMD

Man Years:

Total:	2.00
Professional:	1.50
Other:	.50

Project Description:

Objectives: To isolate and purify specific hormone receptors in particular receptor tissues; to map the function of the receptor against activation of adenyl cyclase; to decipher the putative link between generation of cyclic 3',5'-AMP and solute transport in particular cell systems.

Methods Employed: Polypeptide and catecholamine hormones are purified and iodinated by standard methods or methods developed in the laboratory specifically for that purpose. Rate of association of hormones is determined by incubating plasma membrane fractions or whole cell with labeled hormones. The kinetics of binding is determined by rate measurements as well as assessment of steady state conditions. Standard mathematical analyses of results are performed utilizing double reciprocal or Scatchard plots. Specific hormone binding is defined in terms of dissociation constant (K_m) of the system. Physiologically significant binding shows K_m's compatible with the dose-response relationship recognized for the hormone at the physiological level.

Major Findings: Specific receptors for glucose in the glucose transport system of the isolated renal tubule have been identified. A procedure was developed utilizing radioiodinated phloridzin to identify transport receptors for glucose. Two receptors for phloridzin were found in isolated renal tubules from the renal cortex. One type of receptor is a high affinity system with a K_m of approximately 5×10^{-7} M that is affected by phenols as well as sugars at very high concentrations. The low affinity-high K_m system is stable to boiling. It was also proved in contradistinction to results of other workers, that phloridzin indeed is transported by renal tubular cells.

Incubation of parathyroid hormone with isolated renal tubules for 30 to 60 minutes leads to increased transport of phloridzin. This increase in transport presumably reflects increased synthesis of the carrier or transport system. There is no evidence in this system for immediate activation of transport.

A plasma membrane fraction has been prepared from turkey erythrocytes and this fraction contains adenyl cyclase responsive to catecholamines. The receptor for catecholamines in the plasma membrane of the erythrocytes was studied by determining binding of tritiated isoproterenol to the preparation. Catecholamine binds rapidly to the preparation at 37°; binding is essentially complete within 10 to 12 minutes. This binding is specific for catecholamines; tracer hormone is displaced by isoproterenol, epinephrine, norepinephrine, dopamine and dopa, but not by alpha-adrenergic agonists or antagonist. Treatment of the membranes with trypsin allows release of the receptor from the remainder of the membrane fraction. This "solubilized" receptor shows the same high specificity for catecholamines as the plasma membrane preparation itself. Further characterization and purification of the receptor are in progress.

Additional studies in this laboratory confirm the results of Sutherland and his collaborators that catecholamines cause a rapid increase in cyclic AMP content of the avian erythrocyte. After the intracellular concentration of cyclic AMP reaches a certain point, it begins to diffuse (or possibly is transported) into the medium. Investigators in the NHLI have carried out some studies suggesting that potassium is transported into the avian erythrocyte in response to epinephrine. Studies are in progress in an effort to decipher the link between cyclic AMP generation, movement through the cell and solute transport.

Significance to Biomedical Research and Program of the Instutute: There are a number of disease states which probably represent abnormalities of hormone receptors or the coupling between receptor, generation of cyclic AMP and physiological response to the hormone. This project represents the possible development of a model system wherein the connection between receptor binding of hormone, generation of cyclic AMP and transport can be investigated. Diseases such as nephrogenic diabetes insipidus or pseudohypoparathyroidism can better be explained once the interconnecting links between these cellular events is understood.

Proposed Course of Project: Continuing studies are directed towards isolation of the receptors for particular hormones in receptor cells; solubilization and purification of the receptor; analysis of the inter-connecting substance between binding and adenyl cyclase activation; identification of the system activated by cyclic AMP in stimulating transport of solute.

Honors and Awards: None

Publications: None

Serial No. NIAMD-MDB-6 (c)

1. Metabolic Diseases Branch
2. Section on Physiology and Clinical Nutrition
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Total Energy and Substrate Metabolism: Studies in Health and Diseases

Prevous Serial Number: NIAMD-MDB-5

Principal Investigator: Dr. Robert S. Gordon, Jr.

Other Investigators: Dr. Ronald H. Thompson

Cooperating Units: None

Man Years:
Total: .50
Professional: .25
Other: .25

Project Description:

Objectives: To apply the technique of continuous, extended indirect calorimetry (respiratory gas exchange) and a number of related metabolic and physiologic techniques to the clinical study of energy metabolism, a) to elucidate the mechanisms of normal physiologic response, particularly temperature regulation, to various stimuli including climatic stress, b) to study the influence and mechanism of action on metabolism of a variety of endocrine hormones and drugs, and c) to investigate the characteristics of energy expenditure, energy balance, and aberrations of metabolic processes in a variety of disease conditions.

Methods Employed: Indirect human calorimetry by means of complete continuous expired air analysis in the Metabolic Chamber, metabolic balance determinations, total body heat loss by thermoelectric and radiometric techniques under controlled environmental conditions, and precise measurement of evaporative and metabolic weight loss.

Accomplishments: The facilities of the metabolic chamber have been pre-empted almost entirely this year for studies of sweat gland physiology (q.v). The heat and cold stress capability of the chamber has been used on a few occasions to assist investigators in NIAID to diagnose or study patients with fevers of unknown origin, and investigators in NICHD in evaluating 2 cases of hypothalamic disease and defective temperature regulation.

Significance to Bio-Medical Research and Program of the Institute: Same as for 1969-'70 and 1970-'71.

Proposed Course of Project: Same as for 1969-'70 and 1970-'71.

Honors and Awards: None

Publications:

1. Thompson, R. H., Buskirk, E. R., and Whedon, G. D.: Temperature regulation against cold: effect of induced hyperthyroidism in men and women. J. Appl. Physiol. 31: 740-745, 1971.

Serial No. NIAMD-MDB-7 (c)

1. Metabolic Diseases Branch
2. Section on Physiology and Clinical Nutrition
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Mechanisms of Secretion in Human Eccrine Sweat Glands

Previous Serial Number: NIAMD-MDB-9 (c)

Principal Investigator: Dr. Robert S. Gordon, Jr.

Other Investigators : Dr. Ronald H. Thompson and Mr. Del Thrasher

Cooperating Units : None

Man Years:

Total	: 4.25
Professional	: 1.75
Other	: 2.50

Project Description:

Human sweat glands are minute, but have an extraordinarily high secretory capacity compared to their own weight. Therefore, the sweat gland may be a useful model system in the continuing effort to relate cellular metabolism to mechanisms of secretion. In addition, detailed knowledge of the function of sweat glands may be useful in "human engineering," and in elucidating those diseases, like cystic fibrosis, wich affect sweat gland function.

Methods and Major Findings: During this year studies have centered on the use of internally administered radioisotopes to measure the rate of conversion and/or excretion of substrate from blood into sweat. After the test material has been administered, the subject lies for 1 to 3 hours on a hammock, and is exposed to a hot, dry environment (standard conditions 120°F, 12% relative humidity). Weight loss is recorded by the Brookline metabolic balance described in detail in the 1969-'70 report. At the end of the sweating period, solutes are collected by total body washdown. This wash water is then concentrated and subjected to chemical and radioisotopic analyses.

By giving ^{14}C glucose intravenously and measuring the quantity of ^{14}C lactate secreted in sweat, and comparing this with results after administration of ^{14}C lactate by vein, it has been possible to demonstrate that sweat lactate in normal man is derived from blood glucose, not from blood lactate. These findings have been published.

The effect of several days' starvation on the conversion of ^{14}C glucose given by vein to ^{14}C sweat lactate has also been studied. After starvation, the lactate content of a normal subject's sweat decreases by approximately 20%. This change, though not large, is statistically significant. At the same time the fraction of an intravenously administered dose of labeled glucose, which is accounted for within 90 minutes as sweat lactate, is increased by over 50%. These results indicate that the general conservation of glucose during fasting applies much less to sweat glands than to other body tissues. A manuscript describing these results has been submitted.

The appearance in sweat of ^{14}C urea given by mouth has been studied in seven normal subjects. Immediately after the administration of the tracer urea, the specific activity of sweat urea is approximately two-thirds that of blood urea. This finding, combined with earlier studies by other investigators, strongly indicates that the excess urea contained in sweat has been derived from some source other than blood or extracellular fluid. Extending the experiments of earlier researchers, we have followed the specific activity ratio of blood urea to sweat urea for three days after administration of the tracer. After the first day, the specific activity of sweat urea is significantly higher than that in blood, and the ratio has risen in a few cases to values as high as 4. These findings indicate that the probable source of the excess sweat urea is a delay pool, probably located in the epidermis, and supplied by urea which was in the general circulation 1 to 4 days previously. Thus, it is no longer necessary to entertain the hypothesis that there is passive reabsorption of water in sweat ducts, leaving an excess of urea in the sweat as secreted. These results are being prepared for publication.

Proposed Course of Project: During the coming year we hope to undertake further investigations into the glucose metabolism of sweat glands and its regulation. Initial studies using biopsy material suggest that it may be very difficult to cause sweat gland tissue to metabolize all its stored glycogen; this may indicate that this tissue is relatively insensitive to insulin. The mobilization of glucose to supply sweat glands during starvation may require changes in circulating hormone levels on heat exposure; we hope to measure the effects of heat exposure on the blood levels of insulin, glucagon and growth hormone. Finally, it may be advantageous to resume studies of microdissected sweat glands, which would make possible a direct measurement of the sensitivity of this tissue to hormonal stimuli.

Significance to Bio-Medical Research and Program of the Institute:
The processes whereby exocrine glands elaborate and transport their secretory products are of very general interest and are still imperfectly understood. It is hoped that our studies will throw some additional light on the secretory mechanisms within the sweat gland, which may in turn be useful in interpreting the process of secretion in general. More particularly, since cystic fibrosis, a major area of program concern to NIAMD, is clearly manifest as an abnormality of sweat secretion, we always consider the possibility that further progress in understanding the secretion of normal sweat glands may throw light on the basic inherited defect of cystic fibrosis.

Honors and Awards: None

Publications:

1. Gordon, R. S., Jr., Thompson, R. H., Muenzer, J., and Thrasher, D.: Sweat lactate in man is derived from blood glucose. J. Appl. Physiol. 31: 713-716, 1971.

Serial No. NIAMD-MDB-8 (c)
1. Metabolic Diseases Branch
2. Section on Physiology and Clinical Nutrition
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Morphology and Biochemistry of Platelet Function

Previous Serial Number: None

Principal Investigator: Dr. Michael J. Droller

Other Investigators: Dr. Sidney M. Wolfe

Cooperating Units: None

Man Years:
Total : 2.50
Professional : 1.75
Other : .75

Project Description:

The platelet release reaction is a complex, rapid sequence of events in which platelets release calcium, nucleotides, and other constituents into the surrounding medium in response to agents such as thrombin and collagen. The occurrence of this process in vivo presumably is followed by platelet aggregation and hemostasis.

The present study describes for the first time the pattern of morphological changes associated with this reaction.

The data also challenge an earlier hypothesis that stimulation of platelet function is mediated by a decrease in platelet cyclic AMP levels. In response to thrombin, platelets are found to contain increased, rather than the proposed decreased, concentration of cyclic AMP. This increase may be mediated by the production of increased platelet prostaglandins.

Significance to Bio-Medical Research and Program of the Institute, and Proposed Course of Project: These studies add insight into the mechanisms of platelet function and cell secretion in general. The findings and methods used are to be extended to other cell types in future investigations.

Honors and Awards: None

Publications: None

Serial No. NIAMD-MDB-9(c)
1. Metabolic Diseases Branch
2. Phoenix Clinical Research Section
3. Phoenix, Az.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Diurnal Variation in Biliary Lipid Composition: Role in Cholesterol Gallstone Formation

Previous Serial Number: None

Principal Investigator: Allan L. Metzger, M.D.

Other Investigators: Scott M. Grundy, M.D., Ph.D., Ronald Adler, M.D.

Cooperating Units: Clinical Center, NIAMD Floor, Bethesda, Md.

Man Years:

Total	5	1/12
Professional:	1	4/12
Other:	3	9/12

Project Description:

Objectives: Recent investigation into the pathogenesis of human cholesterol gallstones has focused on the liver as the primary site of deranged metabolism. The principle evidence for this conclusion is 1) the decreased total bile salt pool in subjects with gallstones, and 2) the observation that hepatic bile is more lithogenic (i.e. it contains more cholesterol than can be solubilized by the bile acid and/or phospholipid content) than concomitantly obtained surgical gallbladder bile in subjects with gallstones. We have examined several specimens of bile sampled from the duodenum during the fasting and feeding state in subjects with and without gallstones in order to better understand the role of the enterohepatic circulation in the production of lithogenic bile.

Methods Employed: Fourteen southwestern American Indian females with cholelithiasis, 14 Indians without stones, and 20 Caucasian females without stones were studied. Using a triple-lumen tube, sequential bile samples were obtained as follows: an unstimulated, fasting bile specimen was aspirated from the duodenum (hepatic bile); thereafter, gallbladder contraction was induced via endogenous cholecystokinin release using duodenal infusion of an amino acid preparation, and duodenal contents were aspirated (gallbladder bile); then, 40%-fat formula diets were infused continuously for 12-16 hours, and intestinal contents were continuously and simultaneously aspirated (hepatic bile); after the prolonged perfusion period, formula infusion was discontinued and bile was obtained after several hours fasting (hepatic bile).

Major Findings: In all three groups hepatic bile obtained during pre- and post-infusion periods was more lithogenic than both gallbladder bile and bile collected during the infusion period. The degree of relative bile lithogenicity was greater in Indians than Caucasians. This increased lithogenicity in hepatic bile was due to a relative increase in cholesterol content and decreased bile salt content as compared to gallbladder bile. Since virtually all subjects with or without gallstones secreted lithogenic hepatic bile while fasted, we conclude that this was due to decreased bile salt secretion rate brought about by a physiological interruption in the enterohepatic circulation; in other words, most of the bile salt pool was sequestered in the gallbladder during prolonged fasting, and only small amounts were recycled for secretion into bile.

Significance to Bio-Medical Research and the Program of the Institute: Our findings support the existence of a diurnal variation in human hepatic bile composition which is dependent upon the physiological events of fasting and feeding. Since the secretion of lithogenic bile is postulated as being the initial step in the formation of gallstones, a diurnal change in biliary lipid composition created by the normal function of the enterohepatic circulation may greatly accentuate bile lithogenicity at certain times. If incomplete mixing occurs because of some defect in gallbladder function, the presence of localized areas of lithogenic bile may set the stage for stone formation. Therefore, a combination of both hepatic and gallbladder mechanisms may contribute to the pathogenesis of gallstones.

Honors and Awards: None

Publications: None

Serial No. NIAMD-MDB-10(c)
1. Metabolic Diseases Branch
2. Phoenix Clinical Research Section
3. Phoenix, Az.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Study of Cholesterol Gallstone Formation by the Method of Cholesterol Balance

Previous Serial Number: None

Principal Investigator: Scott M. Grundy, M.D., Ph.D.

Other Investigators: Ronald Adler, M.D., Allan L. Metzger, M.D.

Cooperating Units: None

Man Years:

Total	5 4/12
Professional:	1 7/12
Other:	3 9/12

Project Description:

Recent studies have shown that cholesterol gallstone formation is associated with hepatic secretion of an abnormal bile which contains relative excess of cholesterol with respect to the solubilizing lipids -- bile salts and phospholipids. Clearly this abnormality could be due to decreased bile salt secretion, increased cholesterol secretion, or both. Patients with gallstones have been demonstrated to have significantly smaller bile salt pools than normal patients. Studies in our own laboratory have shown a significant reduction in the hourly hepatic secretion rate of bile salts in gallstone patients. These changes could be due to a diminished synthesis of bile salts from cholesterol or a defective intestinal reabsorption of bile salts in the small intestine, resulting in fecal losses of bile salt which exceed the synthetic capacity of the liver.

We have attempted to resolve these questions by measuring daily synthesis rate of both cholesterol and bile acid using the cholesterol balance technique. The method depends on the measurement of the total daily fecal excretion of cholesterol and bile salts by gas-liquid chromatography. In the steady state, the synthesis of bile salts equals excretion, while the daily synthesis of cholesterol equals the sum of cholesterol and bile salt excretion minus the dietary intake of cholesterol.

Methods Employed: Cholesterol and bile acid synthesis were estimated in four groups of subjects using the cholesterol balance method: 1) Indians with gallstones, including 10 females and 2 males, 2) 6 Indian females without stones, 3) 7 Indian males without stones, and 4) 9 young Caucasian

females without stones. All patients were maintained for at least two weeks on an isocaloric diet containing 40% of calories as fat, 75 mg of cholesterol per 1000 calories and 15 mg of plant sterols per 1000 calories. Four to seven day stool pools collected at the end of the dietary period were analyzed by thin-layer and gas-liquid chromatography for neutral and acidic steroids (cholesterol and bile acid degradation products), and corrected to daily excretion rates by the use of nonabsorbable markers. Body weight was maintained within one kilogram by adjusting caloric intake.

Major Findings: Results to date have shown no significant differences in cholesterol or bile acid synthesis among any of the Indian groups with or without gallstones. On the other hand, there were significant differences in both bile salt and cholesterol synthesis between the Indians with gallstones and the group of young female Caucasian controls. Bile acid excretion was greater in the Indians in spite of the fact that their bile acid secretion rates, measured in another study in this laboratory, are significantly lower than Caucasians. These two findings imply a reduced percentage intestinal reabsorption of bile acid in the Indian patients. It is doubtful however that such a defect could account for the reduced bile acid secretion rates and pool sizes in these patients, since in normal patients much greater daily losses of bile acid, such as occurs with cholestyramine administration, are matched by increased hepatic synthesis, so that no net change in pool size can be detected. We, therefore, propose that a defect in the regulation of bile salt synthesis is present in these patients in addition to a relative decrease in reabsorption.

The Indian patients, both with and without stones, also showed a cholesterol synthesis over twice as high as the Caucasian controls. Since the Caucasian patients were both younger and less overweight than the Indian patients, these factors could explain part of this difference. However, comparison of the Indian data with previous balance studies done on markedly obese Caucasian patients in a similar age group shows that the Indian patients synthesized more cholesterol per kilogram body weight than even these very obese Caucasians. Thus the Indians make more cholesterol than Caucasians, and in the absence of any other route of excretion, more cholesterol must pass through the biliary tract. Yet, in these patients, there is less bile acid available to keep this excess cholesterol in solution. Thus the Indian patients are particularly susceptible to the formation of cholesterol gallstones, as demonstrated by a number of epidemiological studies.

Based on our findings we may speculate that a relative defect exists in the hepatic conversion of cholesterol to bile acid, such that the rate of bile salt synthesis, though slightly elevated in relation to the small pool size, is not adequate to compensate for increased fecal losses. To compound the problem, the negative feedback of bile salt on cholesterol synthesis would be reduced, and consequently allow increased new cholesterol synthesis.

Proposed Course: One of the defects of the present design is the lack of a truly "normal" or ideal control group. Because of the very high incidence of gallstones in the Indian population studied, Indians without existing stones may very well be in a premorbid phase of gallstone disease, i.e. the metabolic defect may precede the appearance of demonstrable stones. Differ-

ences between the study and control group in age and weight also tend to obscure the present results. We propose to study cholesterol and bile acid synthesis in a group of male and female Indians between the ages of 18-21 and compare them to a group of Caucasians similar in every respect except race. The data will be supported by ancillary studies of bile composition and secretion rates to identify those individuals particularly at risk of developing gallstones.

Our present results have raised other questions which have significant implications beyond the gallstone problem. Thus we have shown that the Indian patients have a rate of cholesterol synthesis significantly higher than similarly studied Caucasian patients, yet the Indians are known to have a low serum cholesterol level which does not increase with age, and they have a correspondingly lower prevalence of arteriosclerotic heart disease. Is the Indian more efficient at excreting cholesterol in spite of a higher synthesis rate? Does he thus protect himself against arteriosclerosis at the expense of his biliary tract? Investigations of cholesterol pool size and kinetics in the Indian population are clearly indicated and are being planned at the moment.

Honors and Awards: None

Publications: None

Proposed Course: The above alterations in biliary lipid secretion may be due to several parameters of cholesterol and bile salt metabolism including cholesterol synthesis, bile acid synthesis, and bile acid reabsorption. Future studies will concentrate on these areas in relation to the pathogenesis of gallstones.

Honors and Awards: None

Publications:

1. Grundy, S. M. and Metzger, A. L.: A physiologic method for estimation of hepatic secretion of biliary lipids in man. Gastroenterology (In Press).

Serial No. NIAMD-MDB-11(c)
1. Metabolic Diseases Branch
2. Phoenix Clinical Research Section
3. Phoenix, Az.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Output of Biliary Lipids in Southwestern American Indians with Cholesterol Gallstones

Previous Serial Number: None

Principal Investigator: Scott M. Grundy, M.D., Ph.D.

Other Investigators: Allan L. Metzger, M.D., Ronald Adler, M.D.

Cooperating Units: Clinical Center, NIAMD Floor, Bethesda, Md.

Man Years:
Total: 5 7/12
Professional: 1 10/12
Other: 3 9/12

Project Description:

Objectives: Cholesterol gallstones in man are generally associated with lithogenic bile, i.e. bile which contains more cholesterol than can be solubilized by the bile acid or phospholipid content. Recent work indicates that this abnormal bile is secreted from the liver; however, it is not known whether lithogenic bile is formed because of a decreased secretion of cholesterol, or from a combination of these factors. Therefore, in order to better understand the pathogenesis of gallstones and to more accurately characterize the exact mode of formation of lithogenic bile, we have employed a new technique for the quantitative measurement of the hourly secretion rates of all three biliary lipids--cholesterol, bile acids, and phospholipids.

Methods Employed: 17 southwestern American Indian females with proven gallstones were admitted to the Phoenix Indian Medical Center for volunteer studies before elective cholecystectomy. Findings on these subjects were compared with those from three groups of subjects without stones: 6 Indian women, 9 Indian men, and 13 Caucasian women (7 of these were studied in Bethesda, Md.). After routine and diagnostic laboratory tests the night before the study, participants swallowed a three-lumen polyvinyl tube. Using low-dose, X-ray, the two proximal outlets were positioned in the second portion of the duodenum, adjacent to the ampula of Vater. The third outlet was 10 cm. distally, just beyond the ligament of Treitz. Thereafter, liquid formulas containing 40% fat, carbohydrate, and protein were infused through the most proximal inlet at a rate calculated to provide the subject's daily caloric requirement. β-sitosterol, mixed with formula, was infused at a constant rate while duodenal contents and bile were simultaneously and

continuously aspirated at hourly intervals from the other two outlets for a 12 to 14 hour period. From bile aspirated proximally, biliary lipid composition was determined. Also, hourly outputs of the individual lipids were calculated using marker dilution principles. The details of this method are presently being reported.

Major Findings: 1) The majority of Indian females with stones secreted lithogenic bile during the steady state infusion period. Two of the 13 Indians without stones produced lithogenic bile, but this may represent "pre-stone" disease in a high-risk population. Of significance, none of the Caucasian females secreted lithogenic bile during continuous duodenal infusion. 2) Average biliary outputs (± 2SEM) for all 4 groups are charted below. The results are expressed as absolute values (mg/hr) and corrected to ideal weight (mg/70kg ideal weight/hr). The statistical significance of the differences between each group as compared to Indian women with gallstones is also noted.

Subjects	Cholesterol ± SEM		Bile Acids ± SEM		Phospholipids ± SEM	
	mg/hr	mg/70kgIW/hr	mg/hr	mg/70kgIW/hr	mg/hr	mg/70kgIW/hr
Indian Women with Gallstones	47±4	67±5	440±36	622±48	228±23	317±27
Indian Women without Stones	40±8 (NS)	62±20 (NS)	551±72 (NS)	815±160 (NS)	256±29 (NS)	374±70 (NS)
Indian Men without Stones	48±7 (NS)	51±8 (NS)	1013±101 (p<0.001)	1059±110 (p<0.005)	314±37 (NS)	328±41 (NS)
Caucasian Women without Stones	29±3 (p<0.005)	38±4 (p<0.001)	868±137 (p<0.0005)	1147±187 (p<0.02)	251±39 (NS)	333±58 (NS)

Compared to groups without stones, lithogenic bile in Indian females with gallstones was usually the result of two factors - namely, an increased biliary secretion of cholesterol and an associated decreased biliary secretion of bile acids. Using ancillary sterol balance studies, we have concluded that these alterations in biliary lipid secretion can be accounted for by a defective regulation of conversion of hepatic cholesterol into bile salts.

Significance to Bio-Medical Research: This study provides data on the possible hepatic metabolic defect(s) involved in the pathogenesis of cholesterol gallstone formation in American Indians; whether the presence of lithogenic bile in non-Indians with gallstones can be explained by similar mechanisms remains to be proven using duodenal intubation techniques. Our data support the concept that a decreased total bile salt pool is present in subjects with gallstones, since a decreased biliary secretion of bile salt might result from this abnormality. Furthermore, by expanding the bile salt pool with the oral administration of bile salts, bile can be made less lithogenic - possibly because of an increased secretion of bile salts.

The work of this Branch is directed toward understanding the origin and treatment of human diseases by examining derangements of underlying processes involving cellular metabolism and physiology. Because the gastrointestinal tract is particularly well suited to pursue this goal and because this organ system lends itself to investigative efforts characterized as being at the interface between basic science and clinical investigation, gastroenterology is the primary focus of this Branch's clinical and laboratory activity. In addition, this Branch has maintained its interest in various inborn errors of amino acid metabolism and transport.

Studies of the small intestine

The previously developed solid phase immunoadsorbant technique for measuring the synthesis of immunoglobulins by the small intestinal mucosa *in vitro* has been used to study additional patients with sprue as well as patients with hereditary pancreatitis, cystic fibrosis or other diseases of the small intestine in which the so-called secretory immunoglobulin system may be altered. The demonstration of an increase in the intestinal production of anti-gliadin antibodies following the ingestion of gluten appears to constitute a useful diagnostic test to confirm the presence of gluten-sensitive enteropathy in otherwise ambivalent clinical situations. This observation, coupled with the finding that approximately 90% of patients with gluten-sensitive enteropathy possess a common histocompatibility antigen (HL-A8), strengthens the hypothesis that gluten-sensitive enteropathy is an inherited, immunologically mediated disease. (Z. Myron Falchuk, Warren Strober, N. Rogentine, Clementine Sessoms, Roger L. Gebhard, Leonard Laster, Stephen I. Katz and Mark V. Dahl)

As part of a larger effort to isolate, identify and characterize the agent responsible for nonbacterial gastroenteritis (euphemistically referred to as the "intestinal flu") various absorptive and digestive abnormalities have been documented in patients during the acute phase of the illness. During convalescence all of these abnormalities have returned to normal. (Saul G. Agus, Raphael Dolin, Richard G. Wyatt, Robert M. Chanock, Robert S. Northrup and Julius A. Kasel)

This Branch has also initiated studies designed to explore and characterize the mechanism and cellular site of action of the gastrointestinal hormones (gastrin, secretin and cholecystokinin) as well as investigate these parameters in relevant pathophysiologic states. (Jerry D. Gardner, Roger L. Gebhard and Leonard Laster)

Biochemical aspects of disease

Last year we described the results of the analyses of amino acids in liver tissue from normal subjects and from patients with homocystinuria resulting from cystathionine synthase deficiency. Similar studies are currently being extended to two more accessible cell types: white blood cells obtained from peripheral blood, and fibroblasts grown in tissue culture from skin biopsies. Methods have been developed to obtain purified preparations of

these cells without concomitant loss or leakage of internal amino acids and to subsequently extract and analyze the cellular amino acids. Hopefully these studies will enable us to more readily assess the biochemical changes in tissues from patients with cystathionine synthase deficiency. (S. Harvey Mudd, Jeffery R. Poole, William A. Edwards, B. William Uhlendorf and Leonard Laster)

To characterize in vivo the metabolic significance of differences in cystathionine synthase activities in patients with homocystinuria we have initiated studies of nitrogen and sulfur balance. These studies provide a quantitative measure of the relative degree of cystine dependence in a given patient and should enable us to further characterize the differences between pyridoxine-responsive and pyridoxine-nonresponsive patients with homocystinuria. (S. Harvey Mudd, Jeffery R. Poole, William A. Edwards, B. William Uhlendorf and Leonard Laster)

Studies of membrane function

Our studies of membrane function previously focused on the transport of various substrates across cell membranes and were directed toward characterizing the basic functional components of the transport process and investigating diseases in which altered membrane transport is a significant pathophysiologic or etiologic factor. For example, cation transport across the erythrocyte membrane has been found to be altered in several disease states such as Bartter's syndrome, Liddle's syndrome and cystic fibrosis of the pancreas. We have also examined the transport of hexoses and amino acids across other cell membranes in patients having a primary disorder of membrane transport such as cystinuria or alaninuria. Finally, we have applied our knowledge of renal amino acid transport to the problem of reducing serum concentrations of homocystine in patients with homocystinuria. In addition to our studies on membrane transport we have also begun to explore the broader question of membrane function, i.e. enzymatic activities of the plasma membrane and the characteristics of the interaction between the plasma membrane and various drugs, hormones, bacterial toxins and transported substrates. (Z. Myron Falchuk, Roger L. Gebhard, Saul G. Agus, Lynn Taussig, Jerry D. Gardner and Leonard Laster)

Administrative activities

Dr. Leonard Laster's temporary assignment to the President's Office of Science and Technology continues. He assists Dr. Edward E. David, Jr., the Science Advisor to the President, in policy decisions related to biomedical research and health.

Serial No. NIAMD-DHDB-1(c)
1. Digestive and Hereditary Diseases
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies of the small intestine

Previous Serial Number: SAME

Principal Investigator: Dr. Leonard Laster

Other Investigators: Drs. Saul G. Agus, Z. Myron Falchuk, Jerry D. Gardner, Roger L. Gebhard, Paul Plotz and Lynn Taussig, NIAMD; Mrs. Clementine Sessoms, NIAMD; Drs. G. Nicholas Rogentine and Warren Strober, NCI; Drs. Raphael Dolin, Richard G. Wyatt, Robert M. Chanock, Robert S. Northrup and Julius A. Kasel, NIAID; and Drs. Stephen I. Katz and Mark V. Dahl, Dermatology Service, Walter Reed General Hospital

Cooperating Units: National Cancer Institute
National Institute of Allergy and Infectious Diseases
Arthritis and Rheumatism Branch, NIAMD
Pediatric Metabolism Branch, NIAMD
Dermatology Service, Walter Reed General Hospital

Man Years:

Total:	4.3
Professional:	3.8
Others:	.5

Project Description:

Objectives:

The purpose of this study is to investigate 1) intermediary metabolism of the mucosa of the small intestine, 2) factors which regulate mucosal metabolism, 3) characteristic features of the immune system in the human small intestine, and 4) effects of disease on these various processes.

Methods Employed:

Various techniques for studying tissue metabolism were employed. These include the use of isotopically-labeled compounds *in vitro* and *in vivo*, and methods pertinent to enzymology and immunology.

Major Findings:

1. Studies of the immune mechanism in gluten-sensitive enteropathy. (Z. Myron Falchuk, Warren Strober, G. Nicholas Rogentine and Clementine Sessoms)

To date 7 of 7 patients with gluten-sensitive enteropathy when placed on a gluten-containing diet have shown a significant increase in the in vitro production of IgA and IgM by the small intestinal mucosa. Furthermore, in 6 of these 7 patients this increase in immunoglobulin production is composed in large part of antibodies directed specifically against gliadin (the presumed toxic agent in gluten-sensitive enteropathy).

In an effort to develop an in vitro model for gluten-sensitive enteropathy (no animal model for this disease is known at the present time), a system has been developed for maintaining biopsy specimens of human small intestinal mucosa in organ culture for 48 hours. Biopsy tissue from normal subjects remains stable in terms of gross morphology, histology, enzymatic activity and protein synthetic capacity. Tissue from patients with gluten-sensitive enteropathy in relapse shows significant improvement in mucosal histology and a four-fold increase in brush border enzymatic activity during this 48-hour incubation period. Addition of gliadin to the organ culture medium abolishes this recovery.

Since previous studies have indicated that so-called histocompatibility antigens are related to the immune response system, histocompatibility antigen phenotypes were determined in 26 patients with gluten-sensitive enteropathy. Of these 26 patients 23 (88%) had phenotype HL-A8 in contrast to an incidence of 22% for this phenotype in the normal population.

2. Small intestinal immunology in other gastrointestinal diseases. (Z. Myron Falchuk, Warren Strober, Roger L. Gebhard, Lynn Taussig, Clementine Sessoms, G. Nicholas Rogentine, Stephen I. Katz and Mark V. Dahl)

Studies of IgA synthesis by the intestinal mucosa from patients with cystic fibrosis and from patients with hereditary pancreatitis demonstrated increased intestinal immunoglobulin production in both groups of patients. These findings suggest that the observed increased immunoglobulin production is related to the accompanying pancreatic insufficiency.

A majority of patients with dermatitis herpetiformis have an intestinal histopathologic abnormality which is similar to that seen in gluten-sensitive enteropathy, and approximately two-thirds of patients with this disease have malabsorption. To further explore

the etiology of the intestinal pathology in dermatitis herpetiformis, HL-A phenotypes were determined in 26 patients with dermatitis herpetiformis. Of these patients 58% had phenotype HL-A8 in contrast to an incidence of this phenotype of 22% in the normal population. This increased incidence of HL-A8 in both dermatitis herpetiformis and in gluten-sensitive enteropathy suggests that these two diseases are related genetically.

3. Studies of acute non-bacterial gastroenteritis. (Saul G. Agus, Raphael Dolin, Richard G. Wyatt, Robert M. Chanock, Robert S. Northrup and Julius A. Kasel)

In collaboration with NIAID this Branch has studied intestinal absorption and histology in normal volunteers infected with non-bacterial gastroenteritis. During the acute phase of this self-limited disease there was a significant decrease in fat and xylose absorption. Peroral small intestinal biopsies revealed focal villous flattening, disorganization of the brush border and accumulation of eosinophilic material at the height of the illness. The brush border disaccharidase and alkaline phosphatase activities were significantly lowered at the time of illness. During convalescence all of these parameters returned to normal.

4. Cellular immunity and hepatitis-associated antigen (HAA) positive hepatitis (Saul G. Agus and Paul Plotz)

This Branch has begun to investigate the contribution of cellular immunity to the recovery from or propagation of HAA-positive hepatitis. Studies to date have failed to reveal in vitro lymphocyte stimulation by HAA in patients who have recovered from hepatitis. Future studies will be directed at the acute phase of the hepatitis with serial studies of cellular reactivity.

Significance to Biomedical Research and the Program of NIAMD:

These studies provide information about the role of local intestinal immune mechanisms in the pathophysiology of various disease states and about the regulation of biochemical activities of the intestinal mucosa.

Proposed Course:

These projects will be continued and the findings explored further.

Honors and Awards:

The study of histocompatibility antigen frequencies in patients with gluten-sensitive enteropathy was selected for presentation at the Southern

Sectional Meeting of the American Federation for Clinical Research on January 27, 1972. The same work was also selected for presentation at the National Meeting of the American Federation for Clinical Research in May 1972. The studies of intestinal immunoglobulin production in patients with cystic fibrosis has been selected for presentation at the Annual Meeting of the Society for Pediatric Research in May 1972.

Dr. Laster has continued his temporary assignment as a member of the President's Office of Science and Technology. He is responsible for the development of policy related to biomedical research and health, and assists the Science Advisor to the President.

Publications:

Falchuk, Z. M., Rogentine, G. N., and Strober, W.: Predominance of histocompatibility antigen HL-A8 in patients with gluten-sensitive enteropathy. J. Clin. Invest. 51: In press.

Falchuk, Z. M. and Strober, W.: Increased jejunal immunoglobulin synthesis in patients with nontropical sprue as measured by a solid phase immunoadsorption technique. J. Lab. Clin. Med. 79: 1004-1013, 1972.

Serial No. NIAMD-DHDB-2(c)
1. Digestive and Hereditary Diseases
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Biophysical approaches to the study of human disease

Previous Serial Number: SAME

Principal Investigator: Dr. Leonard Laster

Other Investigators: Dr. A. J. Tousimis, Biodynamics Research Corp., and Drs. Saul G. Agus, Z. Myron Falchuk and Jerry D. Gardner, NIAMD

Cooperating Units: Biodynamics Research Corp. (Contract #PH 43-66-10)

Man Years:

Total:	1.7
Professional:	1.7
Others:	--

Project Description:

Objectives and Methods:

Collaborative studies are in progress with Dr. A. J. Tousimis for the purpose of applying biophysical techniques to the study of biological problems. At this time we are using the small intestine as a model tissue. The techniques involved include high resolution electron microscopy, electron probe microanalysis, and scanning electron microscopy. This work is sponsored under NIH Contract #PH 43-66-10 with Biodynamics Research Corporation ($50,294.16 for FY 1972).

Major Findings:

1) We are studying the use of combined scanning and transmission electron microscopy in the examination of biological tissue specimens. Biopsy specimens from human small intestine are examined directly in the scanning electron microscope and information about the cell surface and internal structure is obtained. 2) An ultrastructural study of giardiasis in the human small intestinal mucosa is under way with emphasis on the scanning electron microscope.

Significance to Biomedical Research and the Program of NIAMD:

These studies are designed to provide insight into the structure and chemical composition of the intestinal mucosa in health and disease.

Proposed Course:

If support for Contract #PH 43-66-10 is continued, all of these studies will be developed further.

Honors and Awards: None

Publications: None

Serial No. NIAMD-DHDB-3(c)
1. Digestive and Hereditary Diseases
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Biochemical aspects of disease

Previous Serial Number: SAME

Principal Investigator: Dr. Leonard Laster

Other Investigators: Drs. Saul G. Agus, Z. Myron Falchuk and Jeffery R. Poole, NIAMD; Mr. William A. Edwards, NIAMD; Dr. S. Harvey Mudd, NIMH; and Dr. William Uhlendorf, DBS

Cooperating Units: National Institute of Mental Health
Division of Biologics Standards

Man Years:

Total:	3.1
Professional:	2.6
Others:	.5

Project Description:

Objectives:

The purpose of this study is to examine pathways of metabolism in tissues of the gastrointestinal tract and the disruption of these pathways in disease.

Methods Employed:

Various techniques for studying tissue metabolism were employed. These included the use of isotopically-labeled compounds *in vitro* and *in vivo* and methods pertinent to enzymology and quantitative analytical biochemistry.

Major Findings:

We have continued our exploration of the biochemical changes associated with cystathionine synthase deficiency (homocystinuria). Studies of the amino acid composition of liver tissue from control patients and patients with homocystinuria due to cystathionine synthase deficiency were initiated last year. As we reported, an accumulation of S-adenosylhomocystine was observed in the liver of cystathionine synthase deficient patients, while none was detected in the liver tissue of normal subjects.

This observation was consistent with current concepts of homocystine metabolism in man. To further extend our understanding of this inborn error of metabolism we have extended similar studies into other areas: in particular, leukocytes obtained from peripheral blood and fibroblasts grown in tissue culture from skin biopsies. Methods have been developed to obtain purified preparations of these cell types without concomitant loss or leakage of internal amino acids and to subsequently extract and analyze their cellular amino acids. (William A. Edwards, Dr. S. Harvey Mudd)

To quantitate differences in cystathionine synthase activities in patients with homocystinuria we have undertaken nitrogen balance studies using low cystine diets with supplemental amino acids. This should provide a quantitative measure of the relative degree of cystine dependence of pyridoxine-responders and nonresponders and further insight into the difference(s) between the two types of patients. (Drs. Jeffery R. Poole and S. Harvey Mudd, and William A. Edwards)

Sulfur balance studies are being conducted on patients with homocystinuria while ingesting a diet having a fixed sulfur composition for the purpose of delineating further the difference between pyridoxine responders and nonresponders. (Drs. Jeffery R. Poole and S. Harvey Mudd, and William A. Edwards)

Using fibroblasts obtained from tissue cultures of skin biopsies from patients with homocystinuria due to various enzyme defects, we are attempting to subclassify homocystinuria both genetically and enzymatically into several distinct entities. (Drs. Jeffery R. Poole, S. Harvey Mudd and William Uhlendorf, and William A. Edwards)

Significance to Biomedical Research and the Program of NIAMD:

These studies extend our insight into approaches to the therapy of cystathionine synthase deficiency and deepen our understanding of the pathophysiology of the disease. In addition these studies should broaden our knowledge of the general question of intermediary metabolism of sulfur-containing amino acids.

Proposed Course:

The general approach exemplified by these studies will be continued.

Honors and Awards: None

Publications: None

Serial No. NIAMD-DHDB-4(c)
1. Digestive and Hereditary Diseases
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies of membrane transport in mammalian tissues

Previous Serial Number: SAME

Principal Investigator: Dr. Jerry D. Gardner

Other Investigators: Drs. Lynn Taussig and Leonard Laster, NIAMD; Drs. Lincoln C. Chen and James M. Boyle, NIAID

Cooperating Units: Pediatric Metabolism Branch, NIAMD
National Institute of Allergy and Infectious Diseases

Man Years:

Total:	1.78
Professional:	1.28
Others:	.5

Project Description:

Objectives:

1) To characterize functionally the mechanism by which various substrates cross the plasma membrane of different mammalian cells; 2) to identify the metabolic and humoral factors which influence the transport of various substrates across the plasma membrane; and 3) to develop techniques which will distinguish between binding of a substrate to the membrane and translocation of the substrate across the membrane.

Methods Employed:

By using slices of intact tissue, isolated cells or isolated plasma membranes, and by using radioactive tracers, we have studied the transmembrane movement of various substrates. Metabolism of the transported substrate was evaluated by use of techniques appropriate to the substrate being studied.

Major Findings:

Our previous studies of sodium influx and outflux in human and rabbit erythrocytes which could be explained by a "two-site model" were extended and elaborated upon. The effect of other monovalent and divalent cations on the translocation of sodium and potassium across the plasma membrane of erythrocytes from various species was explored.

The model for dibasic amino acid transport in human erythrocytes was tested for its applicability to the transport of amino acids in other tissues such as cultured human fibroblasts, rabbit erythrocytes and guinea pig lymph node cells. In general the salient features of the model which we previously proposed to explain the transport of lysine in human erythrocytes were found to be applicable to the transport of dibasic amino acids in these other cell types.

Attempts are under way to isolate various fractions of the plasma membrane from rabbit and hamster intestinal epithelial cells. It is anticipated that these preparations will then be used to explore various enzymatic, metabolic and transport activities of the intestinal epithelial cell.

Significance to Biomedical Research and the Program of NIAMD:

These cellular transport processes are of fundamental importance in all mammalian metabolism so that the overall significance of these studies is broader than the program of any one institute. Furthermore, in order to explain the pathogenesis and/or the etiology of diseases in which a derangement of membrane transport plays a prominent role, it is first necessary to understand as precisely as possible the functional aspects which characterize the movement of a particular substrate across cell membranes.

Proposed Course:

These projects will be continued and the findings explored further.

Honors and Awards: None

Publications:

Gardner, J. D. and Lapey, A.: Sodium outflux and ATPase activity in human and rabbit erythrocytes. J. Applied Physiol. 31: 161-163, 1971.

Shibolet, S. and Gardner, J. D.: Effect of temperature on sodium transport in human erythrocytes in vitro. Amer. J. Physiol. 221: 1358-1360, 1971.

Gardner, J. D. and Levy, A. G.: Transport of dibasic amino acids by human erythrocytes. Metabolism 21: 413-431, 1972.

Gardner, J. D., Shibolet, S., and Ginzler, E. R.: A two-site model for sodium transport in human erythrocytes. Biochem. Biophys. Res. Comm. 45: 1548-1553, 1971.

Gardner, J. D., Shibolet, S., and Ginzler, E. R.: A two-site model for sodium transport in rabbit erythrocytes. Biochem. Biophys. Res. Comm. 46: 1361-1367, 1972.

Serial No. NIAMD-DHDB-5(c)
1. Digestive and Hereditary Diseases
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies of diseases with altered membrane transport

Previous Serial Number: SAME

Principal Investigator: Dr. Jerry D. Gardner

Other Investigators: Drs. Lynn Taussig, Roger L. Gebhard, Z. Myron Falchuk and Leonard Laster, and William A. Edwards, NIAMD

Cooperating Units: Pediatric Metabolism Branch, NIAMD

Man Years:

Total:	1.87
Professional:	1.37
Others:	.5

Project Description:

Objectives:

1) To characterize the mechanism by which the membrane transport of various substrates is altered in certain diseases; 2) to relate these alterations of membrane transport to the pathogenesis and clinical manifestations of the disease.

Methods Employed:

By using slices of intact tissue, isolated cells or isolated plasma membranes, and by using radioactive tracers, we have studied the transmembrane movement of various substrates. Metabolism of the transported substrate was evaluated by using techniques appropriate to the substrate being studied.

Major Findings:

Previous studies from this Branch documented the presence of altered membrane cation transport in erythrocytes from patients with Liddle's syndrome. Studies are in progress to further characterize both functionally and biochemically the alterations in the erythrocyte membrane responsible for the previously observed alterations.

We have previously demonstrated that patients with Bartter's syndrome can be divided into two groups on the basis of whether or not cation transport in their erythrocytes is altered. During the past year we have extended our previous observations to other patients with Bartter's syndrome and have increased the number of patients observed in each group. In addition, we have further documented our previous observation that relatives of patients with Bartter's syndrome tend to have a similar alteration in erythrocyte sodium transport.

Our Branch has been studying two siblings who have diabetes mellitus, diabetes insipidus, optic atrophy, neurogenic bladder, and alaninuria. These patients have normal transport of alanine across the small intestinal mucosa and the erythrocyte plasma membrane in vitro. Studies are in progress to investigate the renal transport of alanine.

Previous studies from this Branch indicated the potential therapeutic efficacy of α-aminoisobutyric acid in lowering the plasma homocystine levels in patients with homocystinuria. The presumed mechanism involves a competition between α-aminoisobutyric acid and homocystine for reabsorption by the renal tubule. These studies have been continued on more patients with homocystinuria. In addition, we have initiated studies of the effect of α-aminoisobutyric acid on the reabsorption of homocystine and other amino acids by the rabbit kidney in vitro.

We have continued our attempts to develop a simple, reproducible bioassay system to evaluate body secretions from patients with cystic fibrosis for their ability to inhibit the transmembrane movement of various substrates. These studies are directed toward finding a simple, reproducible technique for detection of the disease, as well as for detecting the heterozygous carrier state.

Significance to Biomedical Research and the Program of NIAMD:

These cellular transport processes are of fundamental importance in all mammalian metabolism so that the overall significance of these studies is broader than the program of any one institute. Furthermore, in order to explain the pathogenesis and/or the etiology of diseases in which a derangement of membrane transport plays a role, it is first necessary to understand as precisely as possible the functional aspects which characterize the movement of a particular substrate across cell membranes.

Proposed Course:

These projects will be continued and explored further.

Honors and Awards: None

Publications:

Lapey, A. and Gardner, J. D.: Abnormal erythrocyte sodium transport in cystic fibrosis of the pancreas. Pediat. Res. 5: 446-451, 1971.

Gardner, J. D., Lapey, A., Simopoulos, A. P., and Bravo, E. L.: Abnormal membrane sodium transport in Liddle's syndrome. J. Clin. Invest. 50: 2253-2258, 1971.

Gardner, J. D., Simopoulos, A. P., Lapey, L., and Shibolet, S.: Altered membrane sodium transport in Bartter's syndrome. J. Clin. Invest. In press.

Gordon, R. S., Jr., Gardner, J. D., and Kinzie, J. L.: Low mannitol clearance into cholera stool as evidence against filtration as the source of stool fluid. Gastroenterology. In press.

Hadler, N. M., Gill, J. R., Jr., and Gardner, J. D.: Impaired renal tubular secretion of potassium, elevated sweat sodium chloride concentration, and plasma inhibition of erythrocyte sodium outflux as a complication of systemic lupus erythematosus. J. Arthritis and Rheumatism. In press.

The major change in the Branch in 1971-72 was the departure of Dr. Robert Perlman for a post in the Physiology Department of Harvard University Medical School. He was replaced in the Diabetes Section by Dr. Matthew Rechler. Dr. Jesse Roth began a period of duty at Hebrew University, Jerusalem. Foreign workers have included scientists from Italy, France, Hungary and South Africa and active collaborative research between the Branch and the Universities of Rome, Naples and Marseilles has continued.

I. Thyroid Biochemistry

A. Thyroid Cell Membranes.

The first step in TSH action is its binding to the thyroid cell plasma membrane. This interaction and its effect on adenyl cyclase has received further study. Phospholipase A was shown to abolish the TSH response. Phospholipid isolated from thyroid and other tissues restores TSH sensitivity. The nature of the phospholipid involved in this TSH response is under investigation (Wolff).

B. Thyroxine Synthesis.

The model reaction for thyroxine synthesis involving the precursor 4-hydroxy-3,5-diiodophenylpyruvic acid (DIHPPA) has been explored further by investigating certain reaction mechanisms involving this precursor. Photooxidation of DIHPPA produced a compound which upon removal of iodine and alkalinization led to the formation of homogentisic acid (HGA). The nature of the HGA precursor, previously unknown, is now under study (Cahnmann).

C. Iodoproteins.

The number, size and chemical composition of the primary polypeptide chains of thyroglobulin (19S) and a related thyroid iodoprotein (27S) are still under investigation. Complete reduction and alkylation of disulfide bonds and exposure to various dissociating reagents gives a complex product mixture; the smallest units present in significant quantity have molecular weights of 30,000 to 45,000. Several of these have been isolated from 19S and 27S iodoproteins and were shown to have an amino acid composition different from that of the starting protein. No N-terminal amino acid residues could be detected, indicating that these groups are chemically blocked. A simple repeating structure for the thyroglobulin molecule has not been revealed by these studies (Bilstad, Salvatore, Edelhoch, Rall).

D. Thyroid Protein Synthesis.

The defective iodoprotein synthesis in a transplantable rat thyroid tumor (line 1-1c) was shown to be related to a lack of release from thyroid membranes. After radioiodine incorporation, a membrane-iodoprotein complex was isolated from which thyroglobulin could be released by 2% digitonin. After incubation

of tumor slices with N-acetyl-^{3}H-mannosamine, a precursor of sialic acid, no label was found in thyroglobulin whereas normal thyroid rapidly formed labeled thyrogloblin in the soluble phase. It appears likely that sialic acid incorporation is required for thyroglobulin secretion and that the tumor is defective in this step of thyroglobulin synthesis (Monaco, Robbins).

E. Thyroid Hormone Secretion.

Secretion of thyroid hormone is initiated by the engulfment of colloid by apical cell processes and the formation of colloid droplets. This process is blocked by colchicine, a drug which binds to a microtubular protein; this protein has now been isolated from thyroid cells. Agents such as hexylene glycol and D_2O which stabilize microtubules also inhibit secretion. The colchicine block occurs after generation of cyclic AMP. Cytochalasin B, a substance which disrupts microfilaments, also inhibits endocytosis. Therefore, both microtubules and microfilaments are involved in the process of endocytosis in the thyroid cell (Wolff, Straub).

F. Control of Thyroid Function.

The possible usefulness of lithium carbonate in the treatment of hyperthyroidism was investigated further in eight patients with detailed analysis of iodine kinetics. Lithium was shown to decrease thyroid iodine secretion in every subject, a property shared only by iodide. The computer analyses also indicated that lithium decreases peripheral thyroxine disappearance, and this was confirmed by direct experiment. The block of hormone secretion, about 60%, was similar to that achieved with iodine. Lithium appears to be a useful agent in treating severe hyperthyroidism where rapid improvement is desired, but its role in the treatment of thyroid storm cannot be predicted without further study (Temple, Carlson, Wolff, Robbins, Berman).

Thyrotropin-releasing hormone (TRH) is secreted by the hypothalamus and causes thyrotropin release from the pituitary. This substance is now available for clinical trial. Its use in stimulating function in thyroid carcinoma was investigated, employing intravenous infusion of large doses for three days. No functional response was observed despite a significant increase in serum TSH in each case (Sachson, Rosen, Robbins).

In a patient with isolated TSH deficiency, the administration of TRH caused a prompt release of prolactin but not of TSH. This further defines the site of the pituitary defect in this disease (Sachson, Rosen).

G. Thyroid Hormone Transport.

Work has continued on the chemical nature of the thyroid hormone transport proteins of serum. The stability of highly purified human thyroxine-binding prealbumin (TBPA) in urea and guanidine was investigated by fluorescence and absorption techniques. TBPA was minimally affected by 6M guanidine at pH 8 and appears to be the most stable protein yet examined (Branch, Edelhoch, Robbins).

Measurement of the thyroxine-TBPA interaction using the fluorescent probe 1,8-analinonaphthylene sulfonate (ANS) was studied further in order to define the mechanism by which one mole of thyroxine causes the quenching or dissociation of two moles of ANS. Thyroxine appears to displace both ANS molecules. Equilibrium dialysis confirmed a single site for thyroxine and two equivalent sites for ANS, 3,5-diiodophenyl propionic acid and 4-OH-3,5-diiodobenzaldehyde (Pages, Branch, Cahnmann, Edelhoch, Robbins).

5-Dimethylaminonaphthylene sulfonyl chloride (DNS) binds covalently to TBPA in the thyroxine-binding site. Studies are in progress to identify the locus of DNS binding by peptide analysis (Ferguson, Pages, Cahnmann, Robbins).

The unusual protein-protein interaction of TBPA with the retinol binding protein (RBP) has been examined by fluorescence polarization and ultracentrifugation. Contrary to published reports, there appear to be 4 binding sites for RBP (van Jaarsveld, Robbins, Edelhoch).

H. Measurement of Iodocompounds.

Improved methods for detection of ^{127}I in the subnanogram range are under investigation. The use of double-labeled complexes is under study (Lewallen).

I. Thyroid Hormone Action.

The possibility that thyroid hormone might affect the rate of ciliary beating in guinea pig trachea and frog esophagus was explored. A new photoelectric technique was developed, and the classic particle transport method was also used. Thyroxine and triiodothyronine *in vitro* and *in vivo* had no effect on the ciliary beat frequency. A marked accelerating effect of ATP was shared by other nucleotides and appeared related to calcium chelation. Prostaglandin and cyclic AMP also stimulated ciliary beating in the frog (Carlson, Robbins).

The proposition that thyroid hormones act by altering the electrical potential of cell membranes has been investigated in cerbral cortex slices. Thyroid hormone inhibited cyclic AMP enhancement produced by high K^+, histamine and ouabain but not that produced by electrical stimulation. Therefore, the hormone effect appears to be on a process other than membrane depolarization *per se* (Gruenstein, Rall).

II. Polypeptide Hormones

A. Structure.

Analysis of configurational transitions in human growth hormone show that this hormone behaves similarly to bovine growth hormone, human placental lactogen and ovine and bovine prolactin. The three dimensional structure of all of these hormones are, therefore, homologous (Aloj, Edelhoch).

B. Ectopic Hormone Production by Tumors.

Selected low titer antisera exhibit increased binding of labeled hormone in the presence of very small amounts of unlabeled hormone. This "apparent cooperativity" adds to the sensitivity of radioimmunoassay. This assay has confirmed the presence of chorionic gonadotropin (hCG) indistinguishable from normal in bronchogenic, gastric and renal carcinoma and has revealed secretion of the β-subunit of hCG in a pancreatic carcinoma. In both cases, however, the ectopic polypeptide appears to have a higher molecular weight than the normal. A giant-cell tumor of lung has been grown in tissue culture and hCG production has been documented (Rosen, Sachson, Weintraub).

C. Growth Hormone.

Further analysis of a group of 30 patients with acromegaly treated by pituitary radiation has been carried out. After 3 to 6 years (16 patients), plasma growth hormone levels decreased 78%; the level was <10 ng/ml in 2/3 and <5 ng/ml in 1/3. This treatment appears to be as effective as pituitary ablation (Gorden, Roth).

D. Insulin.

In a patient with islet cell tumor a hitherto undescribed form of insulin was found in plasma. It was characterized by exclusion from G50 sephadex greater than that of proinsulin, and by greater biological potency than proinsulin (Gorden, Kahn, Roth).

Insulin stimulates the uptake of α-aminoisobutyric acid (AIB) in rat thymocytes by increasing the rate of influx through an effect on K_m as well as V_{max}. This was abolished by an inhibitor of protein synthesis. Cyclic AMP and prostaglandin also stimulated AIB influx, but the former acted only on V_{max}. Since the insulin effect was additive to that of cyclic AMP or prostaglandin, these agents appear to work through separate mechanisms (Goldfine, Roth, Neville).

Sulfonyl ureas are agents which lower blood sugar. They were found to act as competitive inhibitors of phosphodiesterase in hamster islet-cell tumor as well as in a number of extra-pancreatic tissues. The latter may explain the extrapancreatic actions of these drugs including their detrimental effects on the heart (Goldfine, Roth).

E. Interaction of Polypeptide Hormone with Cellular Receptors.

Further study of the insulin receptors in plasma membranes of rat liver has shown them to be heterogeneous. A high affinity site ($K \simeq 2 \times 10^9$) has a low capacity, and a lower affinity site is present in greater quantity. These membranes also possess a degradation site for insulin. Although specific for insulin structure, inactive insulin analogs are degraded as rapidly as the native molecule. In the obese-hyperglycemic mouse, liver membranes exhibit decreased insulin binding due to a decrease in the

number of receptor sites. This may explain insulin resistance in these mice (Kahn, Gorden, Roth, Neville, Freychet).

Cultured and circulating human lymphocytes have been examined for receptor sites for polypeptide hormones. They have been found to bind insulin as well as growth hormone, the receptors having an affinity and specifity similar to those on liver and fat cells. This finding has provided a means to study the occurrence of altered receptors in human disease. Initial studies have revealed a decreased affinity of insulin receptor sites on circulating lymphocytes in obese subjects, a finding consistent with the insulin resistance seen in these patients. Lymphocytes also exhibit specific receptors for growth hormone, and have been used to establish a radioreceptor assay for human growth hormone (Gavin, Archer, Lesniak, Gorden, Roth, Neville).

An insulin-secreting islet cell tumor in the hamster was used to study glucagon binding since glucagon stimulates insulin release from β-cells. A specific glucagon receptor was found and is suitable for radioreceptor assay (Goldfine, Roth, Neville).

III. Adrenal

Colchicine and other agents, which inhibit hormone secretion in thyroid cells through an action on microtubules, were found to stimulate steroid secretion by adrenal tumor cells in culture. This unexpected effect occurs beyond the step of cAMP enhancement and depends on ongoing protein synthesis (Temple, Wolff).

IV. Protein Structure and Synthesis

Thermal transitions in Bence-Jones proteins have been studied by optical methods. Thermal denaturation increases the β-structure and may be related to the precipitation of these proteins by heat. These proteins are very unusual in that the fluorescence of the two tryptophanyl residues are totally quenched. It is of interest that one tryptophan is in the variable region of the molecule, and the other is in the constant region, since the findings indicate that they have identical environments (Pollet, Edelhoch).

In salmonella typhimurium, an intercistronic region between the hisD and hisC genes, has been shown to exist. By the use of frameshift mutation revertants, enzymes containing variable portions of the hisD, hisC and intercistronic products have been obtained. Analysis of the amino acid sequence of the intercistronic product will be possible by analyzing these fused enzyme complexes (Rechler).

Serial No. NIAMD/CEB 1c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Nonenzymic Model Reaction For The Conversion of 4-Hydroxyphenylpyruvic Acid Into Homogentisic Acid

Previous Serial Number: None

Principal Investigators: H. J. Cahnmann, Ph.D.

Other Investigators: T. Matsuura, Ph.D., A. Nishinaga, Ph.D., I. Saito, Ph.D.

Cooperating Units: Department of Synthetic Chemistry, Faculty of Engineering, Kyoto University, Kyoto, Japan

Man Years:

Total: 1
Professional: 1/2
Other: 1/2

Project Description:

Neither the mechanism by which 4-hydroxyphenylpyruvic acid (HPPA) is converted in vivo into homogentisic acid (HGA) nor any intermediate in this conversion are known.

In order to explore possible reaction mechanisms, photooxygenation of HPPA in the presence of a sensitizer (rose Bengal) was carried out in a weakly alkaline medium where the phenolic hydroxyl of HPPA is at least partially ionized. (The phenolate anion is required for an efficient photooxidation of phenols). In this reaction HPPA was converted into a HGA precursor which yielded HGA when the pH was raised to > 12. The nature of the HGA precursor is now under investigation.

4-Hydroxy-3,5-diiodophenylpyruvic acid (DIHPPA), the diiodinated cogener of HPPA, can be photooxidized at a somewhat lower pH since its phenolic pK is lower. Photooxygenation of DIHPPA led to a compound which upon removal of iodine and alkalinization to pH > 12 also gave HGA.

Honors and Awards: None

Publications: None

Serial No. NIAMD/CEB 2c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Thyroxine-Protein Interactions

Previous Serial Number: CEB 2c and CEB 9c

Principal Investigators: H. J. Cahnmann, Ph.D., H. Edelhoch, Ph.D., R. N. Ferguson, Ph.D., J. Robbins, M.D., R. A. Pages, Ph.D., P. van Jaarsveld, Ph.D.

Other Investigators: M. Brackbill

Cooperating Units: None

Man Years:

Total: 3 3/4
Professional: 2 1/2
Other: 1 1/4

Project Description:

In work carried out last year by W. Branch, H. Edelhoch and J. Robbins, it was found that the fluorescent probe 1,8-anilinonaphthalene sulfonate (ANS) binds to human thyroxine-binding prealbumin (TBPA) and is competitively displaced by thyroxine (T_4) and its analogs. In continuation of this work, several independent methods have been applied to this system in order to clarify the nature of the interaction and to permit quantitative assessment of binding parameters.

Equilibrium dialysis studies have confirmed that at pH 7.4, TBPA has one strong binding site for T_4 and for T_3, but has two equivalent sites for ANS. Also in agreement with results obtained by fluorescence methods, 3,5-diiodo-4-hydroxyphenylpropionic acid shows binding to one site at pH 8.6 and to two sites at pH 5.6. 4-Hydroxy-3,5-diiodo-benzaldehyde shows binding to two equivalent binding sites at pH 8.6.

Ultraviolet spectroscopy also shows that TBPA has two binding sites for ANS. TBPA-bound ANS and free ANS show a positive difference spectrum with a maximum at 383 nm. Addition of T_4 causes a decrease in the difference spectrum which is complete upon addition of 1 mole of T_4 per mole of protein.

5-Dimethylaminonaphthalene sulfonyl chloride (DNS-Cl), a structural analog of ANS, covalently binds to TBPA. TBPA containing 2-4 moles of DNS per mole of protein has been shown to bind minimal quantitites of T_4.

TBPA dansylated in the presence of T_4, followed by removal of T_4, shows almost normal T_4-binding capcity. Peptide maps (chromatography, followed by electrophoresis at pH 3.6) of tryptic digests of DNS-TBPA and TBPA are virtually identical except that one of the spots is strongly fluorescent on the map obtained from DNS-TBPA. This peptide will be investigated further.

Cyanogen bromide cleavage of DNS-TBPA and of TBPA gives a large and a small fragment. CNBr cleavage of DNS-TBPA shows that the DNS is associated with the large fragment. It has been shown by Morgan and co-workers (Biochim. Biophys. Acta 236: 798, 1971) that the single Met in the TBPA monomer is residue 13. Thus, the DNS in our preparation is not N-terminal nor is it coupled to Lys-9.

The interaction between human TBPA and retinol-binding protein (RBP) has been studied by means of fluorescence polarization and ultracentrifugation. Contrary to reports in the literature, TBPA appears to have 4 binding sites for RBP. The details of this interaction are under study.

Honors and Awards: None

Publications:

1. Branch, W. T., Robbins, J. and Edelhoch, H.: Thyroxine-Binding Prealbumin: Conformation in Aqueous Solutions. J. Biol. Chem. 246: 6011-6018, 1971.

2. Robbins, J.: Thyroxine-Binding Proteins in Serum. In Sunderman, F. W. and Sunderman, F. W., Jr. (Eds.): Laboratory Diagnosis in Endocrine Diseases. St. Louis, Mo., Warren H. Greene, 1971, pp. 221.

Serial No. NIAMD/CEB 3c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Nonenzymic Synthesis of Thyroxine and 3,5,3'-Triiodothyronine

Previous Serial Number: CEB 1c

Principal Investigator: H. J. Cahnmann, Ph.D.

Other Investigators: H. Ogawara, Ph.D., J. M. Bilstad, M.D.

Cooperating Units: None

Man Years:

Total: 0
Professional: 0
Other: 0

Project Description:

Inactive

Honors and Awards: None

Publications:

1. Ogawara, H. and Cahnmann, H. J.: Nonenzymic Synthesis of Iodothyronine Residues in Thyroglobulin. Biochim. Biophys. Acta P19: 328, 1972.

2. Ogawara, H., Bilstad, J. M. and Cahnmann, H. J.: Iodoamino Acid Distribution in Thyroglobulin Iodinated In Vivo and In Vitro. Biochim. Biophys. Acta P19: 339, 1972.

Serial No. NIAMD/CEB 4c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Structure of Polypeptide and Protein Hormones

Previous Serial Number: CEB 3c

Principal Investigators: H. Edelhoch, Ph.D., R. Pollet, M.D., S. Aloj, M.D., G. Salvatore, M.D.

Other Investigator: R. Lippoldt

Cooperating Units: None

Man Years:

Total: 4 1/2
Professional: 3 1/4
Other: 1 1/4

Project Description:

The thermal transitions of the molecular conformation of Bence-Jones proteins have been studied by circular dichroism (CD) and fluorescence measurements. An increase in β-structure is observed in the thermally denatured K proteins. The acquisition of β-structure in the thermally denatured protein may be responsible for the well-known high temperature precipitation of this protein. The fluorescence spectra of the Bence-Jones K proteins appear to be unique since they show no tryptophanyl emission.

Since the two tryptophans in the K species are in invariable positions in the variable and constant parts of the molecule, the data support the homology of the variable and constant portions of the molecule. The total quenching of their emission is unique amongst proteins and therefore provides strong presumptive evidence that the two residues are in identical environments.

Analysis of several configurational transitions of human growth hormone by optical criteria revealed similar behavior to the previously examined growth and prolactin hormones, i.e., human chorionic prolactin, ovine and bovine prolactin and bovine growth hormone. These data strongly imply that the three-dimensional structures of the growth and prolactin hormones are homologous.

Studies have been initiated on the structure of human chorionic gonadotropin in order to understand the interactions of its two subunits.

The stability of hyman thyroxine binding protein (TBPA) in urea and guanidine solutions has been investigated by fluorescence and absorption techniques. Contrary to currently held theories of guanidine denaturation, TBPA is almost stable in 6M guanidine at pH 8. The half-life of denaturation is many hours. The rate increases rapidly at higher guanidine concentrations at pH 8.0 or at lower pH values in 6M guanidine. By this criterion, TBPA appears to be the most stable protein yet examined.

Honors and Awards: None

Publications:

1. Aloj, S. M. and Edelhoch, H.: The Molecular Properties of Human Chorionic Somatomammotropin. J. Biol. Chem. 246: 5047-5052, 1971.

2. Covelli, I., van Zyl, A. and Edelhoch, H.: Spectrophotometric Determination of Monoiodotyrosine, Diiodotyrosine and Thyroxine in Iodoproteins. Anal. Biochem. 42: 82-90, 1971.

3. Schneider, A. B. and Edelhoch, H.: Equilibrium Density Centrifugation of Thyroglobulin in RbCl: Effect of Iodine. J. Biol. Chem. 246: 6592-6598, 1971.

4. Cramarossa, L., Andreoli, M., Schneider, A. and Edelhoch, H.: Effect of Freezing in Methylmercaptoimidazole on the Structure of Thyroglobulin. Endocrinology 89: 741-748, 1971.

Serial No. NIAMD/CEB 5c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Measurement of Iodocompounds in Biological Materials

Previous Serial Number: CEB 4c

Principal Investigator: C. G. Lewallen, M.D.

Other Investigators: Han Hoch, Ph.D.

Cooperating Units: Veterans Administration Center, Martinsburg, West Virginia

Man Years:

Total: 1 1/4
Professional: 1
Other: 1/4

Project Description:

In the past year professional activities were expended along the following lines:

1. Service as chairman of the NIH Radiation Committee terminated in August, 1971. Service as Vice Chairman of that committee and Committee membership terminated in March, 1972. Activities were largely the same as described on last year's project report.

2. Work in collaboration with Dr. Hans Hoch on non-protein thyroxine binding factors in human serum has continued. Electrophoretic runs in agar gel containing thyroxine throughout the gel suggest that protein carriers of thyroxine may be identifiable by the change in their electrophoretic mobility in comparison to runs made without added thyroxine. Under these conditions it appears that a beta globulin previously unidentified as a binding protein may act as a thyroxine carrier. The electrophoretic studies in gels of protein binders will be extended to gel diffusion and will include studies of binding factors in protein free serum dialysates.

3. The cerate-arsenite procedure of I^{127} analysis is exquisitely sensitive and can be carried down to the subnanogram range (see Hoch and Lewallen, Clinical Chemistry 15: 204-215, 1969). Its principal short comings are the tedium and complexity of the procedure and the high frequency of interferring factors, often unknown and unpredictable. A project is underway to develop a simpler and more reliable procedure of comparable sensitivity.

Principal attention is being given to double isotope dilution. The following progress has been made:

(a) A suitable preliminary combustion step has been adopted from the literature and proven to be free of significant iodine losses by use of iodine tracer techniques.

(b) A rather extensive literature search has been completed. This indicates the current lack of a simple, reliable substitute for the cerate-arsenite procedures and an extreme paucity of applications of double isotope dilution to this problem in the past.

(c) Attempts to quantitate $I^{127}I$ by measurements of the distributions of multiple iodine labels in 2 phase systems were unsuccessful.

(d) Currently principal attention is being devoted to separation of double labeled complexes of iodine by electrophoretic techniques.

4. A report is in preparation of previous work in which a variety of iodo-compounds of biological interest can be separated by anion exchange chromatography.

Honors and Awards: None

Publications: None

Serial No. NIAMD/CEB 6c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Analysis of an Intercistronic Region in the Histidine Operon of Salmonella Typhimurium

Previous Serial Number: LMB 4

Principal Investigator: M. M. Rechler, M.D.

Other Investigators: C. B. Bruni, M.D., R. G. Martin, M.D.

Cooperating Units: Istituto di Patologia Generale, Università di Napoli, Naples, Italy; Laboratory of Molecular Biology, NIAMD

Man Years:

Total: 1 1/4
Professional: 3/4
Other: 1/2

Project Description:

Analysis of a frameshift mutation near the end of the seond gene in the histidine operon of Salmonella typhimurium, the hisD gene, indicated that an intercistronic region exists between hisD and the succeeding gene, hisC (1). Revertants of this frameshift mutation were obtained in which the hisD and hisC products were covalently fused (2). In order to determine the amino acid sequence which corresponds to the nucleotides of the intercistronic region, mutants were selected which synthesize only a fragment of the hisD-hisC fused enzyme. These contain a complete and functional hisD-enzyme, a protein segment corresponding to the intercistronic region, and the proximal portion of the hisC-enzyme. By comparing the COOH-terminal amino acid sequence of these mutant proteins with that of the wild type hisD-enzyme, it should be possible to deduce the amino acid sequence corresponding to the intercistronic region.

Honors and Awards: None

Publications: None

Serial No. NIAMD/CEB 7c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Action of Thyroid Hormones

Previous Serial Number: CEB 10c

Principal Investigators: H. Carlson, M.D., J. Robbins, M.D.

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 1 1/4
Professional: 3/4
Other: 1/2

Project Description:

Since hormones affect contractile tissues, hormonal effects on ciliated epithelia were studied. In frog esophagus, particle transport methods were initially used; compounds affecting transport were further studied using a photoelectric technique detecting light scintillations reflected from the epithelial surface. Only the photoelectric method was used in studying guinea pig trachea. In esophagus, in vitro, 10 μM thyroxine or triiodothyronine, 1 μM cortisol, progesterone, estradiol or testosterone, and 1-100 μM epinephrine, serotonin, dopamine, isoproterenol or tyramine had no effect; 2 μM prostaglandin E_2 (PGE_2) produced marked cilioacceleration after 1 hour. PGA_1, PGB_1, and $PGF_{2\alpha}$ had slight or no action. 3',5'-dibutyrylcyclic AMP at 0.1-5 mM gave even greater cilioacceleration after a 5-60 minute delay. 1 mM 3',5'-cyclic GMP had minimal or no effect, as did 1 mM 2',3'-cyclicAMP, 10 mM adenosine or guanosine, and 20 mM theophylline. Frog cilia responded within ten seconds to 1-1000 μM ATP, GTP, ITP, CTP and UTP with marked cilioacceleration; diphosphonucleotides at 0.1-1 mM also immediately increased the beat frequency. Monophosphonucleotides at 1 mM had no effect. In guinea pig trachea, no response was seen after in vitro tri-iodothyronine at 10 μM, 10 μM epinephrine, 2 μM PGE_2, PGA_1, or $PGF_{2\alpha}$, 1 mM ATP, or 1-5 mM 3',5'-dibutyrylcyclicAMP. In vivo administration of thyroid hormone or propylthioyracil failed to affect the rate of tracheal ciliary beating.

Honors and Awards: None

Publications: None

Serial No. NIAMD/CEB 8c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Protein Synthesis in the Thyroid Gland

Previous Serial Number: CEB 8c

Principal Investigators: F. Monaco, M.D., J. Robbins, M.D.

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 3/4
Professional: 1/4
Other: 1/2

Project Description:

Previous studies on the radioiodine-labeled proteins of the transplantable rat thyroid tumor line 1-1C2 showed that only 30% of the labeled protein was in the soluble phase (Salabè and Robbins, Biochem. Biophys. Acta 214, 198, 1970). The microsomal fraction contained thyroglobulin-related protein which had a sedimentation coefficient of 12S when solubilized by deoxycholate. The present study was undertaken to investigate the possibility that this represents a defect in the mechanism of thyroglobulin release. Tumors were labeled with ^{125}I *in vitro* for 30 min. to 24 hours in oxygenated Earle's medium, or *in vivo* for 15 min. to 5 days. At the present time the proportion of soluble iodoproteins in this tumor line has decreased to 5-10% of the total radioiodine-labeled proteins, more than 90% of this sedimenting below 8S. Treatment of the insoluble fraction by sonication or vigorous homogenization resulted in the solubilization of 50 ± 10% of the iodoprotein. At all times *in vitro* and at times less than 1 day *in vivo*, the solubilized iodoprotein sedimented as a broad band not separating from the top of a 10-40% sucrose gradient; there was no 19S component. Treatment of this material (or of the original pellet) with 2% digitonin led to the relase of a 19S iodoprotein. This 19S component was tested against an anti rat 19S thyroglobulin serum by double diffusion in agar and showed a reaction of partial identity with native thyroglobulin. These findings indicate that the tumor makes a 19S thyroglobulin-like iodoprotein which is not released into the soluble phase; solubilization by either vigorous homogenization or sonication gives rise to an iodoprotein-membrane complex from which 19S thyroglobulin can be extracted by digitonin.

To investigate the role of glycoprotein synthesis in defective TG secretion we studied incorporation of N-Ac-^{3}H-mannosamine, a precursor of sialic acid. In normal thyroid ^{3}H was incorporated (∿4%) mainly into soluble 19S TG within 1 h. In tumor ^{3}H was incorporated (∿1%) only in a light component. No ^{3}H-TG was seen in "soluble" or "solubilized" protein. To study the first step of carbohydrate incorporation we used N-Ac-^{3}H-glucosamine. In normal thyroid ^{3}H was incorporated (∿2%) more slowly into 19S and "solubilized" protein contained ^{3}H-TG. Tumor incorporation was ∿1/3 the control and ^{3}H was mainly in a 3-8S component and also in a broad peak between 12S and 19S. Thus the tumor has a decreased incorporation of carbohydrate and no apparent incorporation of a sialic acid precursor into TG.

Honors and Awards: None

Publications: None

Serial No. NIAMD/CEB 9c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Ectopic Protein Production by Tumors

Previous Serial Number: CEB 12c

Principal Investigator: S. W. Rosen, Ph.D., M.D.

Other Investigators: R. Sachson, M.D., A. S. Rabson, M.D., I. Stotler, B.S., Y. Kadesky, B. D. Weintraub, M.D., H. Hansen, M.D., H. Sussman, M.D., D. Golde, M.D., N. Papadopoulos, Ph.D.

Cooperating Units: Pathology Dept., Stanford University Medical Center, Palo Alto, California; Pathologic Anatomy Dept., Clinical Center, NIH; Thyroid Unit, Mass. General Hospital, Boston, Massachusetts; Cancer Research Institute, University of California Medical Center, San Francisco, California; Walter Reed Army Institute of Research, Washington, D.C.; NCI Oncology Unit, V. A. Hospital, Washington, D.C.

Man Years:

Total: 1 3/4
Professional: 1 1/2
Other: 1 1/4

Project Description:

Studies continue on the phenomenology of ectopic protein production by tumors - both hormonal and nonhormonal proteins. Detection of such proteins in serum may be pathognomonic of neoplasm.

A. "Apparent Cooperativity"

Studies with a variety of hormone-antihormone systems (hCG, hCS, bTSH) have confirmed the observation of Matsokura et al. for ACTH, that selected low titer antisera display increased binding of labeled hormone in the presence of very small amounts of unlabeled hormone. The "apparent cooperativity" promises to add an additional dimension in radioimmunoassay (RIA). The phenomenology of optimal antiserum production and the molecular mechanisms responsible for the effect are under study.

B. Ectopic Chorionic Gonadotropin (hCG) and Chorionic Somatomammotropin (hCS)

Use of a highly specific guinea pig anti-hCG serum showing this "apparent cooperativity" has confirmed previous studies with less specific antisera that the gonadotropin produced ectopically by 5 patients with neoplasm (4 bronchogenic, gastric carcinoma) is immunochemically indistinguishable from hCG (Stotler). Careful gel chromatography of these sera, however, suggests that the putative hCG behaves with an apparently higher MW than an iodinated reference preparation of purified urinary hCG (Kadesky). We are looking into the adequacy as column markers and RIA standards of conventional reference preparations which may differ from native material by virtue of perturbations in vivo (hepatic, renal degradation), in purification or in iodination.

The ectopic hCG sera are being bioassayed to see whether the differences in bio/immuno ratios found for hCG produced in vitro by clonal lines of choriocarcinoma will also be seen in the case of ectopic hCG production.

Rabson has been successful in growing in vitro a giant-cell carcinoma of the lung from a patient with ectopic hCG production. The in vitro production of hCG by these cells has been documented. Studies are in progress relating to the control of hCG production and secretion by this tumor line.

A patient with renal carcinoma (Golde) has been found to produce hCG and hCS. Although any carcinoma can theoretically produce any protein, gonadotropin production associated with carcinoma of the GU tract has rarely been reported.

The first 100 prospective cases of bronchogenic carcinoma have been collected (Hansen) and we are analyzing sera for the presence of the placental tumor markers hCG and hCS.

C. Ectopic hCG-β

Serum and tumor extracts from a patient with pancreatic carcinoma have been shown to contain a material immunochemically indistinguishable from the beta subunit of hCG (Weintraub). The serum does not contain bioactive gonadotropin, consistent with the absence of bioactivity in hCG-β. Gel chromatography of the serum reveals the peak of immunoactive hCG-β migrating with a higher apparent MW than a reference preparation of purified iodo hCG-β (Kadesky).

D. Ectopic LDH with an Extra Isozyme

Studies continue on a woman with unexplained lactate dehydrogenasemia and an extra LDH isozyme. The clinical data so far support the possibility that she has a cryptic neoplasm making the extra isozyme.

Honors and Awards: None

Publications:

1. Rosen, S. W. and Weintraub, B. D.: Monotropic Increase of Serum FSH Correlated with Low Sperm Count in Young Men with Idiopathic Oligospermia and Aspermia. J. Clin. Endocrinol. 32: 410-415, 1971.

Serial No. NIAMD/CEB 10c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies with Thyrotropin Releasing Hormone (TRH)

Previous Serial Number: None

Principal Investigator: S. W. Rosen, Ph.D., M.D.

Other Investigator: R. Sachson, M.D.

Cooperating Units: None

Man Years:

Total: 1
Professional: 1/2
Other: 1/2

Project Description:

A. Isolated TSH Deficiency

Administration of TRH to a patient with isolated TSH deficiency failed to increase serum TSH from its basal undetectable level (<2.5 μU/ml) but serum prolactin showed the normal prompt rise. This suggests that the TRH receptor on the prolactin-secreting cell is intact and tends to exclude a generalized defect of TRH receptors in this disease (Sachson).

B. TRH and Thyroid Carcinoma

TRH has been administered by continuous IV drip to 3 patients with metastatic functioning thyroid carcinoma. In all cases the tumor metastases were capable of concentrating ^{131}I and the purpose of the study was to attempt to demonstrate an increase in uptake of radioisotope by tumor tissue after TRH administration. No patients showed a functional response to TRH despite the fact that the serum TSH was significanly increased in each case (Sachson).

Honors and Awards: None

Publications: None

Serial No. NIAMD/CEB 11c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Interaction of Insulin with Receptors in Liver

Previous Serial Number: CEB 13c

Principal Investigators: C. R. Kahn, M.D., P. Gorden, M.D., J. Roth, M.D.

Other Investigators: D. M. Neville, Jr., M.D., P. Freychet, M.D.

Cooperating Units: Laboratory of Neurochemistry, NIMH; Hôpital Saint-Antoine, Paris, France

Man Years:

Total: 1 1/4
Professional: 1
Other: 1/4

Project Description:

The first step in insulin action is binding to specific receptors on the plasma membrane of target cells. Previous work here had shown monoiodoinsulin was biologically active and therefore a suitable tracer for studies of insulin interactions with membranes. Using this high specific activity ^{125}I-insulin, plasma membranes of rat liver were shown to possess insulin receptors with the specificity of the biologic receptor.

This interaction of insulin with its receptor in plasma membranes of rat liver has been shown to be more complex than originally believed. The insulin receptor population is not homogeneous, but is composed of at least two types of sites: a high affinity-low capacity and low affinity-high capacity site. The equilibrium constant for the high affinity site is about 2×10^9. Both the affinity constants and the number of receptor sites are influenced by temperature and ionic strength. The dissociation reaction of insulin from its receptor is also consistent with a heterogeneous receptor population. The receptor population is not stable but is undergoing continuous degradation by proteases endogenous to the membranes.

In addition to the binding site, the purified plasma membranes of liver appear to possess a degradation site for insulin. This degradation reaction does not have the specificity of the insulin receptor. Thus, inactive insulin analogues may be degraded as rapidly as the native molecule. The reaction, however, is relatively "specific" for the insulin structure. The K_m for the "insulin specific enzyme" is about 1.7×10^{-7}M.

Most recently, we have examined the insulin-receptor interaction in the obese-hyperglycemic mouse. This animal syndrome is a model of the insulin resistant state often seen in obese patients and adult onset, obese diabetics. The liver membranes of these mice bind only 16-35% as much insulin as those of their thin litter mates. This decrease in binding appears to be the result of a decrease in the number of receptor sites rather than a change in affinity of the receptor.

Honors and Awards: None

Publications:

1. Freychet, P., Roth, J. and Neville, D. M., Jr.: Insulin Receptors in Liver: Specific Binding of ^{125}I-Insulin to the Plasma Membrane and Its Relation to Insulin Bioactivity. Proc. Nat. Acad. Sci. 68: 1833-1837, 1971.

2. Freychet, P., Kahn, C. R., Roth, J. and Neville, D. M., Jr.: Insulin Interactions with Liver Plasma Membranes: Independence of Binding of the Hormone and Its Degradation. J. Biol. Chem., in press.

3. Kahn, C. R., Neville, D. M., Jr., Gorden, P., Freychet, P. and Roth, J.: Defect in the Insulin-Receptor Interaction in Insulin Resistance: Studies in the Obese Hyperglycemic Mouse. Proc. Nat. Acad. Sci., in press.

Serial No. NIAMD/CEB 12c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Intracellular Mechanisms of Insulin Synthesis and Release and Effects of Insulin on Amino Acid Transport in Lymphocytes

Previous Serial Number: CEB 14c

Principal Investigators: I. Goldfine, M.D., J. Roth, M.D.

Other Investigators: D. M. Neville, Jr., M.D., L. Birnbaumer, Ph.D., J. Gardner, M.D.

Cooperating Units: Laboratory of Neurochemistry, NIMH; Laboratory of Nutrition & Endocrinology, NIAMD; Digestive & Hereditary Diseases Branch, NIAMD

Man Years:

Total: 1 1/2
Professional: 1 1/4
Other: 1/4

Project Description:

Glucagon stimulates the beta cell to release insulin both in vitro and in vivo. We studied subcellular fractions of the insulin secreting islet cell tumor of the Syrian (golden) hamster. Tumors were homogenized in 0.001M $NaHCO_3$ and centrifuged. The 10,000xg particles contained adenyl cyclase activity (measured as conversion of $AT^{32}P$ to cyclic $AM^{32}P$) that was stimulated three-fold by glucagon. Other polypeptide hormones, isoproterenol, glucose, arginine and tolbutamide were without effect. Enzyme activity was linear for up to 20 minutes of incubation and proportional to protein concentration. After lyophilization the particles were stable at -20° for several months. ^{125}I-glucagon of high specific activity rapidly bound to the particles. At 0.1 ng/ml, 15% of the ^{125}I-glucagon was displaced; with unlabeled hormones at 100 ng/ml, 75% of labeled hormone was displaced. Secretin, pentagastrin and other polypeptide hormones were without effect. This system permits direct study of the interaction of glucagon with its binding site as well as receptor-cyclase interactions, and also provides the basis for a radioreceptor assay of glucagon.

Sulfonylureas, like tolbutamide, have both pancreatic (insulin secretion) and extrapancreatic effect but the intracellular site of action of these compounds is unknown. In insulin-secreting islet cell tumors of Golden hamsters, cyclic 3',5'-AMP phosphodiesterase was found in particulate and soluble fractions. Tolbutamide and other sulfonylureas acted as competitive inhibitors

of phosphodiesterase with a K_i for tolbutamide of 5×10^{-3}M. The minimum inhibitory concentration of tolbutamide was 0.25×10^{-3}M which is equivalent to effective plasma levels in man. Tolbutamide also inhibited phosphodiesterase in liver, heart, kidney, brain, lung, thyroid and platelets which would explain the sulfonylureas extra pancreatic actions.

We have studied the effect of insulin on rat thymocytes and found that this hormone stimulated the uptake of α aminoisobutyric acid (AIB). Because lymphocytes are a homogeneous population of cells which can be prepared with a minimum of trauma we investigated the nature of this insulin effect. When the kinetics of transport were studied, insulin was found to stimulate AIB influx; outflux was not altered. Insulin acted by lowering the transport K_m and raising the transport V_{max}. The insulin effect was not immediate; rather it increased with time. Cycloheximide, an inhibitor or protein synthesis, inhibited the insulin effect, indicating insulin may have acted by effecting synthesis of AIB carrier protein.

Dibutyryl cyclic AMP also stimulated AIB influx. Unlike insulin dibutyryl cyclic AMP acted only to raise the transport V_{max}. Prostoglandin E_1 (PGE_1) raised intracellular cyclic AMP levels and also stimulated AIB influx.

When insulin was added with either PGE_1 or dibutyryl cyclic AMP, the effect obtained were equal to those seen when each substance was used separately. Insulin neither lowered basal cyclic AMP levels nor blocked the effect of PGE. It was concluded that insulin and cyclic AMP influenced transport through separate mechanisms.

Honors and Awards: None

Publications:

1. Roth, J., Prout, T. E. and Goldfine, I. D., et al.: Sulfonylureas: Effects In Vivo and In Vitro. Annals of Internal Medicine 75: 607, 1971.

2. Goldfine, I. D., Perlman, R. and Roth, J.: Inhibition of Cyclic 3', 5' AMP Phosphodiesterase in Islet Cells and Other Tissues by Tolbutamide. Nature 234: 295, 1971.

3. Goldfine, I. D., Roth, J. and Birnbaumer, L.: Glucagon Receptors in β-Cells: Binding of ^{125}I-Glucagon and Activation of Adenylate Cyclase. J. Biol. Chem. 247: 1211, 1972.

Serial No. NIAMD/CEB 13c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on Human Growth Hormone (HGH) in Man

Previous Serial Number: CEB 16c

Principal Investigators: P. Gorden, M.D., C. R. Kahn, M.D., A. J. Sober, M.D., J. Roth, M.D.

Other Investigators: C. Hendricks

Cooperating Units: None

Man Years:

Total: 2 1/2
Professional: 1 3/4
Other: 3/4

Project Description:

We have reported that in untreated acromegaly plasma GH concentration increases with time and that external irradiation from a conventional supervoltage source is an effective way to reduce GH concentration.

Thirty previously untreated acromegalic patients have been treated with 4000-5000 r supervoltage irradiation and followed for 1-6 years. All 30 patients were restudied at the end of 1-2 years and 16/30 were studied again at the end of 3-6 years.

Prior to treatment mean GH for 30 patients was 58 ng/ml, median 29 and range 7-220 ng/ml. By 1-2 years after irradiation there was a 65% reduction in plasma GH for the 30 patients (mean GH = 22 ng/ml, median 14 and range 3-85 ng/ml). Further reduction in plasma GH occurred with time and by 3-6 years post irradiation there was a 78% reduction in plasma GH for 16 patients (mean GH = 13 ng/ml, median 7 and range 3-50 ng/ml). In these 16 patients GH was less than 10 ng/ml in 2/3 of the group and less than 5 ng/ml in 1/3 of the group.

Three additional patients treated by transfrontal hypophysectomy had a 71, 49 and 10% reduction in GH and when subsequently treated by pituitary irradiation had a further 70, 50 and 47% reduction in GH respectively.

There are wide variations in benefit among individual patients with all modalities of therapy; the long term results of supervoltage irradiation is comparable to the reported results of other forms of irradiation or surgical therapy and is accompanied by trivial morbidity and no mortality.

In conjunction with determining the long term effects of irradiation on plasma growth hormone concentration, we have also studied the effect of elevated growth hormone on the cardiovascular system and have attempted to develop methods of diagnosing systemic disease in patients with acromegaly.

Approximately 20-30% of acromegalic patients are hypertensive and we have found that cardiac disease (arrhymia or failure) can usually be ascribed to hypertension or another known cause of heart disease such as thyrotoxicosis. These patients have had congestive heart failure which has been unexplained by known etiologies. In one of these patients a spontaneous pituitary infarction with reduction of GH to undetectable levels resulted in marked amelioration of the CHF. These patients appear to have arteriosclerotic heart disease. It is, therefore, clear that elevated growth hormone concentrations have important circulatory effects that adversly affect the diseased heart but it is as yet unclear whether growth hormone has direct effects on the heart.

We have found that all acromegalic patients have increased organ weights but clinical organomegaly as determined by palpation is indicative of a second disease state.

Honors and Awards: None

Publications:

1. Gorden, P. and Roth, J.: Clinical Application of the Radioimmunoassay of Growth Hormone. In Sunderman, F. W. and Sunderman, F. W., Jr. (Eds.): Laboratory Diagnosis of Endocrine Diseases. St Louis, Mo., Warren H. Green, Inc., 1971, pp. 200.

2. Gorden, P. and Roth, J.: Assay of Human Growth Hormone in Plasma. In Sunderman, F. W. and Sunderman, F. W., Jr. (Eds.): Laboratory Diagnosis of Endocrine Diseases. St. Louis, Mo., Warren H. Green, Inc., 1971, pp. 205.

3. Gorden, P. and Roth, J.: Efficacy of Conventional Pituitary Irradiation in Acromegaly: 5 Year Follow-Up. Second International Symposium on Growth Hormone, International Congress Seriea #236, Excerpta Medica, pp. 65.

Serial No. NIAMD/CEB 14c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Nature of Plasma Insulin in Man

Previous Serial Number: CEB 17c

Principal Investigators: P. Gorden, M.D., C. R. Kahn, M.D., J. Roth, M.D.

Other Investigators: C. Hendricks

Cooperating Units: None

Man Years:

Total: 1 1/4
Professional: 3/4
Other. 1/2

Project Description:

Big insulin (proinsulin-like component) represents a high proportion of circulating insulin in patients with islet cell tumors (26-80% of total basal insulin). We studied a patient with islet cell carcinoma in whom 80% of circulating insulin is a heretofore undescribed form (tumor big insulin). When plasma from this patient was filtered on G-50 Sephadex, most of the plasma insulin immunoreactivity was recovered as a discrete peak ahead of both non-tumor big insulin and ^{125}I-proinsulin. In all previous studies of plasma from tumor and non-tumor subjects by us and others, all insulin immunoreactivity is recovered with proinsulin or beyond. Using an antiserum that does not distinguish between dilutions of porcine insulin, proinsulin, or non-tumor human big and little insulin, the tumor big insulin had a markedly different reactivity over a 100-fold range of dilutions. Tumor big insulin purified from plasma had 5.5 μU of bioactivity (glucose oxidation in isolated fat cells) per ng of immunoreactivity which is 3-fold greater than that of porcine proinsulin or non-tumor big insulin. Tumor big insulin was converted normally by exposure to trypsin. Release of tumor big insulin was not inhibited by large doses of diazoxide.

Little insulin from this patient's plasma had normal mobility on G-25 Sephadex and was as active as porcine insulin in fat cells, but had the abnormal immunoreactivity found for the patient's big insulin.

It is likely that the proinsulin produced by this tumor is larger in size than heretofore described proinsulin-like substances and has one or more amino acid substitutions in the insulin part of the molecule.

This is the first demonstration in a human of a distinctive insulin.

Studies are underway at present to further define the proinsulin-like component of circulating insulin and to study the interaction of the insulin and proinsulin component with purified liver membranes of rat liver.

Honors and Awards: None

Publications:

1. Gorden, P., Sherman, B. and Roth, J.: Proinsulin-Like Component of Circulating Insulin in the Basal State and in Patients and Hamsters with Islet Cell Tumors. J. Clin. Invest. 50: 2113-2122, 1971.

2. Gorden, P., Freychet, P. and Nankin, H.: A Unique Form of Circulating Insulin in Human Islet Cell Carcinoma. J. Clin. Endocrin. 33: 983-987, 1971.

3. Gorden, P., Roth, J., Freychet, P. and Kahn, R.: The Circulating Proinsulin-Like Components. Diabetes, in press.

Serial No. NIAMD/CEB 15c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Interaction of Polypeptide Hormones with Their Receptors in Target Tissues

Previous Serial Number: CEB 13c

Principal Investigators: J. R. Gavin, III, M.D., M. Lesniak, J. Archer, M.D., P. Gorden, M.D., J. Roth, M.D.

Other Investigators: C. Hendricks, D. Buell, M.D.

Cooperating Units: General Laboratories & Clinics, NCI

Man Years:

Total: 2 1/2
Professional: 1 1/4
Other: 1 1/4

Project Description:

Since polypeptide hormones exert their effects on specific cell receptors, the accessibility of the circulating cell makes this tissue desirable for studies of hormone interactions in man. Using biologically-active monoiodoinsulin, we find that circulating and cultured human lymphocytes have receptors for insulin that have the same affinity and biologic specificity for insulin as the receptors studied in liver and fat cells of rats.

Peripheral lymphocytes have been used to study directly insulin-receptor interactions in normal individuals and in patients with conditions characterized by alterations in insulin sensitivity. To study insulin binding, lymphocytes were incubated with ^{125}I-insulin at 5×10^{-12} - 8×10^{-11} M. The cells were separated by centrifugation and the radioactivity bound to the cell pellet was determined. In the patient studies, the parameters measured were the maximal binding of ^{125}I-insulin per 70×10^{6} cells (B_{max}) and the concentration of unlabeled hormone required for half-maximal inhibition of ^{125}I-insulin binding (ng/ml) to the cells ($1/2B_{max}$). These parameters were chosen in order to compare data from individual patients. To date, no significant difference is observed in the B_{max} of diabetics, acromegalics, obese patients or hypopituitary patients when compared to the normal range. On the other hand, with respect to 1/2 B_{max}, over 50% of the values in the obese group fall outside the normal range, and these differences are highly statistically significant. The exact meaning of this difference is not known. However,

since the $1/2B_{max}$ is a function of the affinity constant of the insulin-receptor interaction, these data suggest that the insulin insensitivity in obesity may result in part from an alteration in the affinity constant of the receptor.

The cultured human lymphocytes contain insulin binding sites with apparent affinity constants of 1.2×10^{10} and 1.45×10^{10} L/M. The presence of the high affinity sites provided a means for the development of a sensitive radioreceptor assay to measure plasma insulin components. With this method the purified insulin component if circulating insulin has the same biologic specificity as pancreatic porcine insulin, whereas the proinsulin-like component has a similar biologic specificity to porcine proinsulin.

Specific growth hormone (HGH) receptors are also present in human cultured lymphocytes. ^{125}I-HGH is bound to these cells and displaced only by biologically active HGH. Furthermore, the ability of an HGH preparation to displace ^{125}I-HGH from the lymphocyte receptor is directly proportional to the biological activity of the preparation; highly purified HGH displaces strongly whereas human placental lactogen (HPL) displaces weakly, consistent with its low HGH-like activity.

Using the cultured lymphocyte, a radioreceptor assy for measuring plasma growth hormone has been developed. Immunoreactive growth hormone from an acromegalic patient's plasma displaced ^{125}I-HGH from the cultured lymphocytes in a manner indistinguishable from a purified pituitary HGH.

These data suggest that it is possible to study directly insulin-receptor interactions in man using peripheral lymphocytes. The data further suggests that it is possible to measure circulating insulin components and growth hormone using the cultured human lymphocyte.

Honors and Awards: None

Publications:

1. Gavin, J. R., III, Roth, J., Jen, P. and Freychet, P.: Insulin Receptors in Human Circulating Cells and Fibroblasts. Proc. Nat. Acad. Sci. 69: 747-751, 1972.

Serial No. NIAMD/CEB 16c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Mechanism of Thyroid Hormone Action

Previous Serial Number: CEB 7c

Principal Investigators: E. Gruenstein, Ph.D., J. E. Rall, Ph.D., M.D.

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 3/4
Professional: 1/2
Other: 1/4

Project Description:

Alteration of the electrical properties of cell membranes has recently been proposed as a mechanism of thyroid hormone action (Gruenstein and Wynn, J. Theoret. Biol. 26, 343, 1970). In order to investigate this theory further we have measured the interaction of thyroid hormone with membrane depolarizing agents in slices of cerebral cortex. This has been done by monitoring cyclic AMP which is known to increase as a function of membrane depolarization (Shimizu, Creveling and Daly, Proc. Nat. Acad. Sci. 65, 1033, 1970). Using the ^{14}C-adenosine prelabeling technique of these workers, thyroid hormone was found to inhibit the cyclic AMP stimulation due to high K^+, histamine, ouabain, and veratridine by as much as 40%, 12%, 14% and 24% respectively. This antagonistic effect of thyroid hormone was apparent within 20 minutes at hormone concentrations of 10^{-6} M. On the other hand, when total endogenous cyclic AMP was measured by the Gilman assay, no effect of thyroid hormone on K^+ stimulation was observed, suggesting that the hormone may be acting on a small localized intracellular pool.

Depolarization of the membranes by direct application of electrical current also caused an increase in cyclic AMP levels, but this stimulation was not affected by thyroid hormone regardless of the cyclic AMP assay used. This suggests that the thyroid inhibition of cyclic AMP stimulation by the chemical agents listed above is in fact due to some ancillary interaction with the membrane not directly involving depolarization. Furthermore, the thyroid hormone inhibitory effect on cAMP levels cannot be accounted for by increased levels of phosphodiesterase since the hormone has been shown to inhibit phosphodiesterase activity slightly.

Honors and Awards: None

Publications: None

Serial No. NIAMD/CEB 17c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Thyroid Iodoproteins

Previous Serial Number: CEB 6c

Principal Investigators: J. Bilstad, M.D., H. Edelhoch, Ph.D., G. Salvatore, M.D., J. E. Rall, Ph.D., M.D.

Other Investigator: R. Lippoldt

Cooperating Units: Office of the Director, NIAMD

Man Years:

Total: 1 3/4
Professional: 1 1/2
Other: 1/4

Project Description:

The goal of this project is to determine the number, size and chemical composition of the primary polypeptide chains of the thyroid iodoproteins, 19S thyroglobulin (MW 660,000) and the 27S iodoprotein (MW ∿1,300,000; generally considered to be the dimer of 19S). This information is necessary to determine the role of the structure of the iodoproteins in the formation of the thyroid hormones, thyroxine and triiodothyronine.

The iodoproteins from hog and bovine thyroids were purified by ammonium sulfate precipitation and agarose gel filtration. In the presence of dissociating agents normally iodinated 19S dissociates into a 12S species (MW 330,000), the smallest non-covalently bound subunit. Following complete reduction and alkylation of the disulfide bonds, which should allow separation of the primary polypeptide chains in the presence of dissociating agents, considerable size heterogeneity was noted. When analyzed by gel electrophoresis in the presence of the detergent, SDS, a significant amount of the 12S species was seen as well as approximately 10 smaller components with molecular weights as low as 35,000 and 10,000. These 2 small components were isolated by gel filtration, initially in the presence of SDS followed by gel filtration in the absence of SDS. Amino acid analyses revealed significant differences compared to native 19S. Further investigation of these fractions is in progress.

Since components as large as 330,000 MW are still found following complete reduction and alkylation, aggregation seems likely. Conditions for dissociation have been tested with limited success including variations in the pH and ionic strength, and the use of agents such as urea plus. isopropanol, guanidine plus propionic acid and lithium diiodosalycylate.

In the presence of 70% formic acid there is a gradual disappearance of the heavier components with the appearance of a component of 45,000 MW which becomes the predominant band in SDS electrophoresis. This component was isolated by gel filtration, and N-terminal analysis by Edman degradation revealed no N-terminal amino residues, apparently due to blocking. Larger amounts of this component are currently being purified for further characterization.

Preliminary studies on human thyroglobulin from normal and hyperthyroid glands also reveal a complex pattern in SDS gel electrophoresis following reduction of the disulfide bonds, and suggest there is no significant difference between these thyroglobulins.

The 27S iodoprotein from bovine and hog thyroid glands when incubated for prolonged periods at 4°C and neutral pH resulted in the formation of 19S and 2.5S species (31,000 MW) which were isolated by gel filtration. Native 19S showed no such dissociation. The iodine content of the 2.5S species was higher than that of 27S and the amino acid content was significantly different. In the presence of 6M guanidine HCl hog 27S dissociated completely into 2 species which were isolated by sucrose density gradient centrifugation and found to have sedimentation coefficients of 2.6S (44,000 MW) and 4.9S (65,000 to 85,000 MW). The appearance of approximately 10 new amino terminal groups on analysis of incubated 27S suggests that peptide bonds were cleaved. Freshly prepared hog 27S was also found to be more sensitive to tryptic digestion than 19S. Since only a limited number of discrete species were formed during the incubation of 27S, there appear to be preferential sites of cleavage suggesting the existence of intra-chain repeating homologous sequences in the 27S iodoprotein.

Honors and Awards: None

Publications:

1. Bilstad, J. M., Edelhoch, H., Lippoldt, R., Rall, J. E. and Salvatore, G.: Isolation and Characterization of Discrete Fragments of 27S Thyroid Iodoprotein. Arch. Biochem. Biophys., in press.

Serial No. NIAMD/CEB 18c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Antimitotic Agents and Adrenal Secretion

Previous Serial Number: None

Principal Investigators: R. Temple, M.D., J. Wolff, M.D., Ph.D.

Other Investigators: None

Man Years:

Total: 1
Professional: 3/4
Other: 1/4

Project Description:

Microtubules have recently been implicated in the secretion of hormones from a number of endocrine glands. In all these glands there is bulk storage of the hormone in the form of granules or colloid, and secretion is thought to involve phagocytic translocation of the stored material. Colchicine also interferes with granule movement in certain non-endocrine tissues. The hypothesis that microtubules promote secretion of stored granular material, and that colchicine and related agents interfere with this process, was therefore tested in several secreting systems.

Microtubular concentrations of colchicine, vinblastine or podophyllotoxin stimulate steroid secretion by Y-1 adrenal tumor cells in culture to an extent that equals a maximal ACTH response. This is in marked contrast to thyroid secretion, which is inhibited at these concentrations, and to TSH secretion by pituitary glands or amylase secretion by parotid glands, which are not affected. Unlike the ACTH response, the stimulation by antimitotic agents is not immediate but commences after a lag of about 6 hours. Thereafter, steroid secretion proceeds at a constant rate for at least 18 hours and this stimulated rate is approximately equal to the maximum rate obtainable with ACTH. The maximum steroid response to ACTH is not further enhanced by colchicine. The steroid products formed after stimulation with the three antimitotic agents are identical to those produced after ACTH stimulation. In contrast to ACTH, the antimitotic agents do not stimulate adenylate cyclase in vitro. Low concentrations of cycloheximide cause comparable inhibition of ACTH or vinblastine-stimulated steroid secretion. It is suggested that the antimitotic agents stimulate steroid secretion at a post-adenylate cyclase step and that this stimulation is dependent on protein synthesis.

Honors and Awards: None

Publications:

1. Temple, R., Williams, J. A., Wilber, J. F. and Wolff, J.: Colchicine and Hormone Secretion. Biochem. Biophys. Res. Commun. 46: 1454, 1972.

Serial No. NIAMD/CEB 19c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies in Thyroid Disease

Previous Serial Number: CEB 11c and CEB 22c

Principal Investigators: R. Temple, M.D., J. Wolff, M.D., Ph.D., J. Robbins, M.D.

Other Investigators: H. Carlson, M.D., M. Berman, Ph.D., D. Murphy, M.D.

Cooperating Units: Mathematics Research, NIAMD; Behavioral Research, NIMH

Man Years:

Total: 1 3/4
Professional: 1 1/2
Other: 1/4

Project Description:

Since lithium has been shown to inhibit release of iodine from the thyroid, we have investigated its therapeutic potential in thyrotoxicosis. Eight detailed ^{131}I kinetic studies were performed on seven thyrotoxic women and data was analyzed using a computer program. Lithium at serum levels of about 1 meq/L decreased the loss of ^{131}I from the thyroid, led to a fall in serum ^{131}I levels and diminished urinary ^{131}I excretion. Computer simulation of the lithium effect required, in every case, that lithium inhibit hormonal and non-hormonal thyroid iodine release. In five cases a second lithium effect was required for a satisfactory fit of the model solution with observed data: namely, an inhibition of hormone disappearance from serum. This has been confirmed by direct measurement of thyroxine disappearance in hyperthyroid subjects.

Neither inhibition of release nor of hormone disappearance seemed to be affected by methimazole (release: 52% decrease without methimazole, 60% with methimazole; hormone disappearance: ~60% decrease in both). When Li^+ was discontinued, recovery of the iodine release rate and hormone disappearance rate over the observed time span was variable, ranging from no recovery to rates that exceeded pre-Li^+ values.

When Li^+ is used alone its effect on serum hormone levels is diminished due to continued accumulation of iodide by the thyroid. Thus, serum thyroxine-iodine levels fell 21 to 30% in 6-8 days in patients who did not receive methimazole and 23 to 59% in 5 days in the methimazole-treated

subjects. For prolonged therapy, therefore, a thiocarbamide drug must be used in conjunction with Li^+. Although potentially toxic, lithium is not difficult to administer in a hospital environment. Because of the lack of information concerning lithium toxicity in extremely ill patients, we cannot at the present time recommend that lithium be substituted for iodide in treatment of thyroid storm.

The effect of lithium on thyroid function in patients with manic depressive psychosis is also under study. A method using two iodine isotopes, one labeling the thyroid and the other labeling the circulating thyroxine, is used since it is a sensitive indicator of changes in thyroid hormone secretion, capable of detecting diurnal variation in secretion rate. Abrupt changes in thyroid iodine secretion are seen after the onset and cessation of lithium administration.

Honors and Awards: None

Publications:

1. Robbins, J.: Hyperthyroidism. In Conn, H. (Ed.): Current Therapy. Philadelphia, Saunders, 1972, pp. 453.

Serial No. NIAMD/CEB 20c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Thyroid Plasma Membranes

Previous Serial Number: CEB 18c

Principal Investigator: J. Wolff, M.D., Ph.D.

Other Investigator: G. H. Cook

Cooperating Units: None

Man Years:

Total: 1 1/2
Professional: 1/4
Other: 1 1/4

Project Description:

Studies on purified beef thyroid membranes adenylate cyclase have been continued. It has been shown that pure phospholipase A abolishes the ability of the receptor to respond to TSH. Crude thyroid phospholipid and purified portions from other tissue restore TSH sensitivity. The nature of the phospholipid is currently being investigated. It has also been found that low concentrations of purine nucleotide triphosphates enhance both basal and TSH-stimulated cyclase activity and also increases the ratio of TSH/basal activity. Similar effects are exerted on stimulation by prostaglandin E_1. The relation of these changes to hormone binding is under investigation.

Honors and Awards: None

Publications:

1. Wolff, J. and Jones, A. B.: The Purification of Bovine Thyroid Plasma Membranes and the Properties of Membrane-Bound Cyclase. J. Biol. Chem. 246: 3939, 1971.

Serial No. NIAMD/CEB 21c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Thyroid Hormone Secretion

Previous Serial Number: CEB 23c

Principal Investigator: J. Wolff, M.D., Ph.D.

Other Investigator: M. Straub, M.D.

Cooperating Units: None

Man Years:

Total: 1 1/2
Professional: 1 1/4
Other: 1/4

Project Description:

The role of microtubules in the thyrotropin or cyclic AMP-stimulated accumulation of cytoplasmic colloid droplets and secretion of iodine from the mouse thyroid gland has been investigated by means of different classes of agents that affect the stability of microtubules. The onset of inhibition of secretion by colchicine, the uptake of ^{3}H-colchicine by thyroid lobes and the binding of ^{3}H-colchicine to thyroidal soluble protein are shown to have similar time courses. Colloid droplet accumulation is also inhibited and is not readily reversed by removal of colchicine from the medium. This appears to be due to the slow washout of the drug ($t_{1/2}$ ~7 hours).

Thyroids contain a soluble colchicine binding protein that resembles microtubule proteins of other tissues with respect to apparent K_m for colchicine, pH optimum and stability characteristics. Colchicine analogs inhibit iodine secretion and colchicine binding in a parallel manner as a function of their antimitotic potencies. Microtubule stabilizing agents such as hexylene glycol and D_2O also inhibit secretion. Thus inhibition of thyroid secretion by antimitotic agents appears to be mediated by an affect on microtubules.

The inhibitory locus of colchicine inhibition occurs after the generation of cyclic AMP. Since stimulation of secretion by this nucleotide is blocked by colchicine whereas TSH-induced accumulation of cyclic AMP is not affected. The disappearance of colloid droplets upon removal of cyclic AMP occurs with a half-life of ~15 min and is not influenced by colchicine. Since low concentrations of colchicine cause total disappearance of microtubules by

electronmicroscopic examination, we conclude that the functioning microtubules appear to play a role in the induction of colloid endocytosis and not in the subsequent fate of the colloid droplet. The purification of "microtubular" protein from beef thyroid tissue is in progress. In addition we have found marked activation of colchicine binding to brain "microtubular" protein by polyvalent inorganic or organic anions.

We have also studied the effect of the mold antibiotic cytochalasin B_1 which disrupts microfilaments. The addition in vitro of cytochalasin B at concentrations of 0.5-3.0 µg/ml to prelabeled mouse thyroid glands interferes with release of iodine derived from thyroglobulin and blocks colloid endocytosis. It is suggested that cytoplasmic microfilaments are involved in the secretory process.

The metabolic effects of Li^+ were compared to those of colchicine since both agents block secretion for the thyroid gland.

Li^+ and colchicine were studied for their effects on glucose oxidation in this tissue. Both 2-25 mM Li^+ and colchicine ($3x10^{-6}$M or more) enhanced the thyrotropin (TSH)-stimulated oxidation of [I-^{14}C] glucose in beef thyroid slices. Lithium also potentiated the TSH-stimulated oxidation of [6-^{14}C] glucose and [I-^{14}C] pyruvate as well as the incorporation of ^{32}P into phospholipid. Colchicine, however, was without effect on [6-^{14}C] glucose and pyruvate oxidation. Methods were adapted to permit measurement of [I-^{14}C] glucose oxidation by mouse thyroid lobes. Neither Li^+ nor colchicine had a significant effect on basal or TSH-stimulated glucose oxidation in this species at concentrations which almost totally abolished secretion. In dog thyroid slices also, TSH-stimulated $^{14}CO_2$ production was unaffected by Li^+ and colchicine at levels which inhibited intracellular colloid droplet formation. The results provide evidence for Li^+- and colchicine-induced dissociation of the responses to TSH and suggested a lack of direct coupling between hormone secretion and glucose oxidation.

Honors and Awards: None

Publications:

1. Williams, J. A. and Wolff, J.: Cytochalasin B Inhibits Thyroid Secretion. Biochem. Biophys. Res. Commun. 44: 412, 1971.

2. Berens, S. C., Williams, J. A. and Wolff, J.: Dissociation of Thyrotropin-Stimulated Hormone Secretion and Glucose Oxidation in Thyroid Glands by Lithium and Colchicine. Biochim. Biophys. Acta 252: 314, 1971.

Serial No. NIAMD/CEB 22c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Role of Cyclic 3',5'-AMP in E. coli

Principal Investigator: R. Perlman, M.D., Ph.D.

Other Investigators: M. Emmer, M.D., M. Straub, M.D.

Cooperating Units: Laboratory of Molecular Biology, NCI

Man Years:

Total: 0
Professional: 0
Other: 0

Project Description:

Terminated

Honors and Awards: None

Publications: None

Serial No. NIAMD/CEB 23c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Assays of Vasopressin

Previous Serial Number: CEB 15c

Principal Investigators: E. Thompson, M.D., C. Beardwell, M.D., J. Roth, M.D.
P. Gorden, M.D.

Other Investigators: M. Lesniak

Cooperating Units: None

Man Years:

Total: 0
Professional: 0
Other: 0

Project Description:

Terminated

Honors and Awards: None

Publications: None

PEDIATRIC METABOLISM BRANCH

Objects of investigation in the past year have been the generalized inherited disease, cystic fibrosis of the pancreas (CF), and familial hereditary pancreatitis (HP).

A. CYSTIC FIBROSIS OF THE PANCREAS (CF)

1. Physiological and Biochemical Studies

Flow rates and electrolytes in minor salivary gland saliva in normal subjects and patients with CF: A new method for extracting saliva from small salivary glands from the inner aspect of the upper lip was devised. This method allows for the first time determination of sodium, potassium, calcium, and magnesium, as well as flow rates. Ten normal adults, 18 to 20 years of age, and 15 patients with CF, 11 to 28 years old, were included in this study.

Sodium was found markedly elevated and flow rates moderately decreased in patients with CF, whereas the other ions determined were equal to those of normal controls. In both groups sodium increased with increasing flow rates, but conversely potassium, magnesium, and calcium decreased inversely to the rates of flow.

The results described in this study confirm our previous findings that it is the small salivary glands that are affected by the electrolyte defect of CF and not the submaxillary and parotid glands in whose secretions sodium levels are normal. It is notable that minor salivary glands are the only exocrine gland system in addition to the eccrine sweat glands that consistently manifest the sodium abnormality in CF. In addition, as shown by the normal values for calcium in the patients tested in this study, the sodium defect appears to be independent of the increased concentration of calcium, as recently postulated for sweat glands by other authors. (Drs. di Sant'Agnese, Pallavicini, NIAMD)

Effects of saliva and plasma from CF patients on membrane transport *in vitro*: Previous studies indicate that some CF body fluids possess the ability to alter the movement of molecules across cell membranes. Attempts to develop simple biological systems have produced various results. We have investigated the effects of mixed saliva and plasma from CF patients on transport of sodium, alanine, and in separate experiments sugar in patients with CF of both sexes, 12 to 28 years of age, and controls of both sexes, age 18 to 32 years.

The effect of the supernatant from centrifuged saliva on the mucosal-to-serosal flux of ^{24}Na across the rat jejunum *in vitro* and on sodium influx and fractional sodium outflux in normal human erythrocytes were studied. Concentrations of sodium and potassium in the saliva specimens were adjusted to 140 mM and 7 mM respectively. The effect of non-centrifuged saliva on the uptake of ^{14}C-alanine by the rat jejunum *in vitro* was determined; electrolyte composition was not adjusted. Finally, the effects of plasma on

^{14}C-3-O-methyl-glucose uptake by the rat jejunum were assessed. There were no significant effects of saliva or plasma from patients with CF on any of the transport phenomena studied.

Additional preliminary studies also indicate that CF saliva, fibroblast homogenates, and tissue culture media from fibroblast cultures do not affect resting transmembrane potentials or short-circuit current across frog skin membranes *in vitro*.

In our systems, therefore, we could not demonstrate any transport-inhibiting effect of CF saliva or plasma. Our results also are not in agreement with previous reports that CF saliva inhibits alanine transport and CF plasma arbutin uptake by the rat jejunum. (Drs. Taussig, Gardner, Kattwinkel, NIAMD)

Jejunal immunoglobulin A (IgA) synthesis in CF: Immunologic abnormalities have recently been described in patients with CF. Accordingly, we have evaluated local IgA production by jejunal mucosa *in vitro* in 7 patients with CF, 7 patients with HP, and 29 normal subjects. IgA synthesis in biopsy specimens obtained perorally was assessed by measuring ^{14}C-L-leucine incorporation into IgA. Incorporated counts binding to specific anti-IgA antibodies covalently linked to bromacetyl cellulose constituted a measure of IgA production. The patients with CF were 14.5 to 41 years of age and their Shwachman scores ranged from 52 to 88. The patients with HP were 14 to 44 years of age. Pancreatic enzymes were absent in all CF patients and in 2 of 7 HP patients; in the remaining 5 HP patients enzyme levels were significantly reduced to 20% of normal.

The mean IgA incorporation value in CF patients was 20373 ± 9244 cpm/mg protein (mean ± SD), and in HP patients was 11403 ± 4585, both values significantly greater than normal (6688 ± 2449) ($p < 0.001$). In addition, the mean IgA incorporation value in CF patients was greater than the value in HP patients ($p < 0.05$). Three of 7 biopsies from CF patients revealed increased numbers of plasma cells on histological examination. There was no correlation between IgA incorporation values and serum IgA levels, age, Shwachman score, severity of lung disease, type of bacteria in sputum cultures, or roentgenographic abnormalities of the small bowel.

The most reasonable explanation for these findings is that patients with CF and HP are under an increased local antigenic stimulus, perhaps due to an altered gastrointestinal microenvironment which leads to increased local IgA production. (Drs. Falchuk, Taussig, NIAMD)

Tissue culture studies: In view of our previous reports (Pallavicini et al., J. Pediat. 77:280, 1970) concerning increased glycogen stores by CF fibroblasts, we have established facilities for growing fibroblasts in tissue culture. In an effort to investigate this phenomenon we are examining glucose uptake and utilization by the cells. This involves timed experiments with 3-O-methyl-glucose to determine the rate of glucose uptake by the cell membrane. We are looking at comparative uptake and diffusion of this compound, the effect of insulin and fetal calf serum on glucose transport, energy

requirements of the cell during the course of the experiments (i.e. Do varying rates of uptake merely reflect varying amounts of stored glycogen at t = 0?).

Preliminary results disclose that CF fibroblasts utilize 3-0-methyl-glucose at a faster rate than normal cells. These results are consistent and seem not to be due to a "trapping" phenomenon (as tested by ^{14}C inulin) or to a simple difference in passive diffusion across the membrane (as tested by the ability of "cold" glucose to suppress uptake). Enzyme activity as related to glucose metabolism will also be investigated. All of the above experiments must be controlled for cell age as increased glycogen in CF fibroblasts is not seen prior to 10 days after inoculation. Further experiments will involve investigation of the relationship of glucose to sodium transport with an eye to relating the increased glycogen to the sodium transport abnormality of CF.

We are also engaged in cooperative studies with Dr. Alexander Spock to investigate the effect of fibroblast sonicates and media on ciliary beat by rabbit trachea; morphologic studies of fibroblasts by electron microscopy with Dr. Samuel Spicer; investigation of methylation of RNA by CF and normal fibroblasts with Dr. Owen Rennert. (Drs. Pallavicini, Kattwinkel, Taussig, di Sant'Agnese, NIAMD; Dr. Spock, Duke Univ. Med. Cen., Durham, N.C.; Dr. Spicer, Med. Col. of S.C., Charleston; Dr. Rennert, Univ. of Fla. Col. of Med., Gainesville)

2. Diagnostic Studies

Isoenzymes of alkaline phosphatase (AP) and related serologic liver function studies in normal adolescence and in CF: Serologic tests for liver function in children have been difficult to interpret because of the lack of good normal data in this age group. AP in particular is known to increase markedly during adolescence secondary to active bone growth, thus making interpretation of this test as a measure of liver function most difficult. An automated technique has been developed requiring 10 microliters of serum to examine total AP and its l-phenylalanine and urea stable components. This technique has been utilized as well as conventional methods for measuring 4 other serological parameters of liver disease: transaminases (SGOT, SGPT), 5' nucleotidase (5'N), and γ-glutamyl transpeptidase (γGTP). The sera of 91 normal controls (age 4-30) and 31 patients with CF were examined.

It is well known that patients with CF may develop hepatic biliary cirrhosis which is often undetectable prior to postmortem examination but may lead to significant clinical problems during life. Our results disclose that total AP is a poor indicator of minimal biliary cirrhosis in childhood because of the masking effect of bone isoenzyme; normal values may exceed by a factor of 2 those previously reported. Determination of the liver fraction of AP, however, renders the test age-independent and unmasks a significant number of abnormal values. Of 31 CF patients studied, 10 had abnormal liver fractions while only 2 had abnormal total APs when compared with age-matched norms. Eight of these 10 patients also demonstrated slightly to markedly elevated transaminases, 5'N, and/or γGTP.

In this study new normal data have been established for several serologic liver function studies in the 4 to 30 age group. Separation of AP isoenzymes discloses a normal peak of bone fraction during adolescence and distinguishes those patients with a significantly elevated liver fraction during puberty. It is felt that AP during adolescence is of minimal value without concomitant isoenzyme determination and that, conversely, the liver fraction of AP can be a good determinant of early biliary cirrhosis in CF. (Drs. Kattwinkel, Taussig, NIAMD; Dr. Statland, Clin. Path., Clin. Cen.)

Human amniotic fluid (AF) Isoamylase: An investigation of the amylase concentration and isoenzyme pattern in AF was performed since the source of the AF amylase has been in doubt. Amylase isoenzyme patterns in human AF have not been examined previously. Twenty AF samples were obtained at various times during gestation by amniocentesis or abortion and at delivery by transvaginal needle aspiration. Homogenized placenta, neonatal urine and serum, adult serum, duodenal fluid, and parotid saliva, and adult homogenized liver were also studied. Total AF amylase concentration was measured by an amyloclastic method. Disc polyacrylamide gel electrophoresis was used to separate the isoamylases and the various bands were identified by squeezing the enzyme-containing polyacrylamide gel column against a starch agar film.. The isoenzymes of amylase were allowed to react with the starch substrate: 15 min. at room temperature for saliva and duodenal fluid and 6 hrs. at 37°C in a moist box for urine, serum, placenta, liver, and AF. After incubation, the zymograms on the starch slides were developed in an iodine solution. The concentration of AF amylase rises significantly during gestation and may be as high as 248 Somogyi units/100 ml at term. The AF amylase isoenzyme pattern was nearly identical to that of neonatal urine and revealed distinct salivary (at least 5 bands) and pancreatic (at least 1 band) components. Isoamylase patterns from homogenized placenta were similar to that of adult serum and different from AF. The amount of amylase in placenta is low and suggests that it is of maternal origin. Maternal proteins (except for IgG) are not known to cross the placenta in appreciable amounts. The similarity of the AF and neonatal urine patterns indicate fetal origin of AF amylase. In contrast to previous beliefs, it appears that the pancreas and salivary glands of the fetus function early in intrauterine life (as early as 16 weeks).

It is planned to apply these findings to study of the time of appearance of pancreatic deficiency in utero in cystic fibrosis and if possible to antenatal diagnosis of this disease. (Dr. Wolf, NIDR: Dr. Taussig, NIAMD)

3. Metabolic Studies

Calcium metabolism and parathyroid function in patients with CF: The studies of calcium metabolism were continued. Five patients with CF underwent calcium infusion tests for parathyroid function: the results were consistent with a state of hyperparathyroidism, as previously reported. In two of these patients parathyroid hormone determinations revealed high normal values which failed to decrease at the end of the calcium infusion despite an increase of serum calcium.

To further delineate the abnormality, the responsiveness of bone and kidney to parathyroid extract (PTE) and the rate of calcium absorption were studied in the five patients. Gastrointestinal absorption of calcium was assessed by the measurement of forearm radioactivity in a large-volume liquid scintillation counter following separate oral and intravenous doses of $^{47}CaCl_2$: it was within the normal range for all subjects despite the presence of malabsorption.

The data indicate that the response to PTE administration in patients with CF does not differ from that of normal subjects, indicating a normal end organ responsiveness. However, the secretion of parathyroid hormone does not suppress normally and is independent of the control of serum calcium. (Dr. Simopoulos, Guest Worker, Drs. Taussig, di Sant'Agnese, Kattwinkel, NIAMD; Dr. Bartter, NHLI; Dr. Murad, Univ. of Va., Charlottesville; Dr. Arnaud, Mayo Clinic, Rochester, Minn.)

4. Clinical Studies of CF

Fertility in males with CF: Normal semen specimens were obtained from two unrelated CF male patients, both meeting all criteria for the diagnosis. Semen from Case #1 (age 26) revealed normal seminal volume, sperm count, sperm motility and morphology, pH, citric acid and fructose concentrations. Case #2's (age 24½) semen also had a normal sperm count and seminal volume; he fathered two children. Blood group analysis was consistent with paternity. These findings are in contrast to the prevailing opinion that virtually all postpubescent males with CF are infertile.

The sterility found in most CF patients is due to aspermia and a low volume of ejaculate, secondary to abnormal, atretic or absent epididymides, vasa deferentia, and seminal vesicles (all mesonephric derivatives). In aspermic CF patients semen is primarily derived from prostatic secretions as shown by its acidic pH, high citric acid and acid phosphatase content and conversely by the low or absent fructose. The normal urinary tracts in CF patients indicate that when infertility occurs, mesonephric development proceeds normally until the 10th to 12th week of gestation. Maldevelopment may occur at variable times thereafter in fetal life or even after birth and the variety and type of findings on pathologic examination suggest it may be secondary to obstruction of structures by abnormal secretions.

Therefore, fertility occurs in a small but significant percentage of male patients with CF and it is mandatory to evaluate the semen from all postpubescent males with CF prior to counseling. (Drs. Taussig, di Sant'Agnese, Kattwinkel, NIAMD; Dr. Lobeck, Univ. of Wis., Madison; Dr. Ackerman, Univ. of Calif., L.A.)

Roentgenographic abnormalities of the duodenum and small bowel in CF: Roentgenographic abnormalities of the duodenum and small bowel in CF have not been well-documented previously. Thirty-three patients with CF, age 4 to 24 years, and 7 with hereditary pancreatitis (HP) were examined with barium esophagogram, upper gastrointestinal series, and small bowel roentgenograms;

in addition 4 patients with CF had hypotonic duodenography. Biopsies of the proximal jejunum were done on 10 CF patients and all HP patients. X-rays of the duodenum and small bowel were scored separately on a 0 to 4 scale, without knowledge of the patients' histories. Eighty-four percent of the patients with CF had roentgenographic abnormalities of the duodenum, 61% with moderate to severe changes. The abnormalities consisted of markedly thickened folds, filling defects, mucosal smudging, and dilatation and redundancy of the duodenum. There was no correlation between the duodenal x-ray findings and age, sex, weight percentile, Shwachman score, severity of lung disease, abdominal pain, history of intussusception or volvulus, hypoalbuminemia, amount of supplemental pancreatic enzymes being taken, pancreatic insufficiency as determined by duodenal intubations (22 patients), or histological material obtained from biopsies and autopsies (2 patients). The etiology of duodenal changes in CF is obscure. All patients with HP had pancreatic deficiency as determined by duodenal intubations and 72-hour stool pools for fecal fat, but x-rays of their duodenums were normal (5 cases) or showed slightly thickened folds (2 cases).

Nine patients (27%) with CF had abnormalities of the jejunum consisting mostly of thickened or coarse folds. Only rarely were findings compatible with a "deficiency" pattern noted; dilatation and segmentation and flocculation of the barium were observed in 2 cases and prolonged transit time was evident in only one patient. Three patients with HP also had minor small bowel changes. (Drs. Taussig, di Sant'Agnese, NIAMD; Dr. Saldino, Diag. Rad., Clin. Cen.)

Prognostic score and clinical evaluation sheet for CF: The improved life expectancy of patients with CF has been associated with an increased frequency of certain complications, some of which have considerable prognostic significance. Pulmonary parameters rather than nutritional and dietary aspects currently appear to be associated with prognosis. A need has arisen for a systematized scoring system based as much as possible on objective criteria; such a system could be used to prognosticate and as a uniform and consistent method of evaluating and following patients for clinical purposes and studies. Previous scoring systems have been more subjective and have not considered these newer complications.

We have devised a new, easily-used system for patients with CF. Seventy-three patients and their x-rays were evaluated independently by two of us. The age range was 5 to 30 years; 40 were male, 33 female. Three and 6-year survival rates were calculated and correlated with the components of the score sheet to determine which factors could be related directly with prognosis.

The system, as derived, contains 2 parts: 1) The Prognostic Score involves primarily pulmonary parameters and totals 100 points. Seventy-five of these are concerned with chest x-ray, pulmonary function tests (V.C. and FEV_1), pulmonary exacerbations, pneumothorax, hemoptysis, pulmonary surgery, cor pulmonale, physical examination of the lungs, clubbing, and cough and sputum production. The remaining 25 points involve nutritional status, activity, and patient attitude toward his disease. 2) The Clinical Evaluation Section does not affect prognostic index and considers other common complications and additional tests not easily obtained on routine clinic visits.

We feel that this system supplies information not provided by existing methods. It allows for registering and appropriately weighing various parameters in a more objective manner and provides the clinician with a concise summary of an individual patient's clinical course and complications. The physician newly introduced to CF may quickly assess in a systematized manner a patient's history, course, and prognosis. Finally, it allows for easy categorization of patients for accurate patient comparison for research purposes. (Drs. Taussig, Kattwinkel, di Sant'Agnese, NIAMD; Dr. Friedewald, NHLI)

Treatment of malabsorption - arginine and sodium bicarbonate: It has been reported that orally administered L-arginine is beneficial in decreasing the amount of steatorrhea, abdominal pain, and improving weight gain in patients with CF. It also has been suggested that oral administration of bicarbonate ($NaHCO_3$) may improve the malabsorption associated with pancreatic insufficiency due to other causes. In view of these findings, a study was undertaken to investigate the efficacy of orally administered L-arginine and $NaHCO_3$ in the treatment of malabsorption due to CF.

Ten CF patients, ranging in age from 11 to 24 years, and with stable pulmonary disease were studied. The absence of proteases in the duodenal fluid was confirmed by duodenal intubation within 18 months of the study. A 100 g fat diet was ordered and actual fat and nitrogen intake was computed daily by a dietitian. Stools were collected in 72-hour pools and 2 or 3 pools were obtained for each period. The study consisted of 4 phases in most of the patients: No supplement, arginine alone, Cotazym alone, Cotazym plus $NaHCO_3$.

The results disclosed that in contrast to previous reports, in the patients studied, L-arginine produced no improvement in the amount of steatorrhea or azotorrhea. In addition, all patients on arginine lost body weight (mean = 1.0 kg) in contrast to the weight gain observed on all other regimens. Although 4 of 8 patients showed improvement in these parameters when $NaHCO_3$ was added to Cotazym alone, the mean improvement for all patients was not statistically significant. (Drs. Kattwinkel, Agus, Taussig, di Sant'Agnese, NIAMD)

Emotional adjustment of adolescents and young adults with CF: As more patients with CF are reaching adulthood, it has become important to assess psychological as well as physical performance and to determine how these patients can best be helped to function well. Therefore, an in-depth psychiatric evaluation was made of adolescents and young adults with CF. Twenty-seven patients, ages 13 to 30, had psychiatric interviews, intelligence tests, and projective psychological testing. Fourteen patients were adolescents, ages 13 to 18, and thirteen were young adults, ages 19 to 30. In 21 families the patient's mother was seen and interviewed. Evaluations were made as to the patient's functioning at work and school, self-esteem, personal relationships, and coping with illness.

These patients have been well able to compensate for physical defects in terms of achievement. However, infantilizing relationships with mothers, withdrawal of the fathers, and denial of the emotional impact of the illness contribute to psychological difficulties in adult life. The question of psychiatric support for these patients and their families needs to be investigated

further. Possible considerations are group therapy for parents and for spouses of CF patients. (Drs. Boyle, Guest Worker, di Sant'Agnese, NIAMD; Ms. Sack, Geo. Wash. Univ., D.C.; Dr. Millican, Georgetown Univ. Hosp., Wash., D.C.)

B. FAMILIAL HEREDITARY PANCREATITIS (HP)

Familial hereditary pancreatitis (HP) is a recently described autosomal dominant disease characterized by recurrent episodes of acute pancreatitis resulting eventually in pancreatic deficiency, pancreatic calcifications, and intestinal malabsorption, usually in early adult life. Seventy-nine patients from 18 families have been reported. All kindreds have been Caucasian and all but two from the United States.

We have studied three additional large kindreds located in a small area of Tennessee, West Virginia, and Virginia respectively. Although the families reside within an area of less than 200 miles in diameter, we have been unable to establish definite inter-family relationships. With 30 definite cases of pancreatitis, the present kindreds represent more than one-third of the total previously described cases. In the families under study other metabolic defects have been excluded by detailed studies of pancreatic and parathyroid function, serum lipids, pathologic specimens, and urinary amino acid content.

HP, although similar in many ways to the adult picture, has many distinctive features: lack of sex incidence, onset usually in early childhood, rarity of alcoholism and gall stones, early onset of calcifications, relative rarity of diabetes mellitus, and good life expectancy.

It is evident, therefore, that at least in the middle eastern section of the United States, hereditary pancreatitis is not a rare disease and usually presents in the childhood age group. HP, indeed, is the most common cause of relapsing pancreatitis in childhood and should always be considered in the differential diagnosis of recurrent abdominal pain and abdominal calcification in the pediatric age group. Contrary to classical teaching, the existence of chronic inflammation of the pancreas with calcification appears to predispose the resultant pancreatic malignancy late in adult life.

While the etiology and pathogenesis of HP are still a mystery, the autosomal dominant type of transmission suggests that the basic defect is not an inborn error of metabolism, but a structural defect. The aminoaciduria reported in two of the five original HP kindreds was probably due to coexistence of HP in incompletely recessive cystinuria. (Drs. Kattwinkel, Lapey, di Sant'Agnese, Pallavicini, Laster, NIAMD)

Serial No. NIAMDD-PMB-1c
1. Pediatric Metabolism
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Metabolic, Physiological, and Biochemical Studies in Cystic Fibrosis.

Previous Serial No.: None

Principal Investigator: Paul A. di Sant'Agnese, M.D.

Other Investigators: Lynn M. Taussig, M.D., John Kattwinkel, M.D., J. Charles Pallavicini, Ph.D., Jerry D. Gardner, M.D., Myron Falchuk, M.D., NIAMDD; Robert O. Wolf, D.D.S., NIDR.

Cooperating Units: Digestive and Hereditary Diseases Branch, NIAMDD; Oral Medicine and Surgery Branch, NIDR; Alexander Spock, M.D., Duke University Medical Center; Samuel S. Spicer, M.D. Medical College of South Carolina; Owen M. Rennert, M.D., University of Florida College of Medicine.

Man Years: Total: 2.75
Professional: 1.5
Other: 1.25

Project Description:

Objectives:

1. The basic defect in cystic fibrosis remains unknown. Recently several investigators have described the presence of two factors in saliva, sweat, and serum of patients: a ciliary inhibitory factor and a factor which inhibits sodium transport across various membranes. Since there is no easy, reproducible assay for either factor, studies were done in an attempt to develop a biologic assay for the sodium inhibitory factor.

2. Several recent reports by other investigators have implied that the basic abnormality in CF may be related to the immunologic system, particularly IgA. Studies were done to determine if IgA production by intestinal mucosa was related to pancreatic deficiency. If it is, then it may not be related to the primary etiologic cause of CF. Therefore, patients with CF and hereditary pancreatitis (both with pancreatic insufficiency) were studied.

3. As of now there is no method available to make a prenatal diagnosis of CF which would be of obvious practical importance in the control of the

disease. Over the past few years amniotic fluid has become a valuable source of material for the prenatal diagnosis of various congenital diseases, but nothing is known about the fluid surrounding fetuses who have a high probabil- of having CF. Ninety percent of patients with CF have pancreatic insufficiency at birth. Studies were done to determine the source and time of appearance of human amniotic fluid amylase isoenzymes.

4. In view of our previous reports (Pallavicini, et al., J. Pediat. 77: 280, 1970) concerning increased glycogen stores by CF fibroblasts, an attempt was made to investigate glucose uptake and utilization by fibroblasts from CF patients and normals. Questions to be answered with fibroblast experiments include: 1) what are the comparative uptake and diffusion of glucose by the cell; 2) what are the effects of insulin and fetal calf serum on glucose transport; 3) what changes of stored glycogen occur during the life cycle of the cell; 4) are there morphologic differences between CF and normal cells; 5) is the "CF factor" present either within the cell or in the growth-supporting media; 6) can other biochemical abnormalities (e.g. methylation of RNA) be demonstrated in the CF cell?

5. Previous studies in our laboratory have shown that it is the small salivary glands that are affected by the electrolyte defect of CF and not the submaxillary and parotid glands. To further characterize these abnormalities the flow rates were measured in minor salivary gland saliva.

Methods Employed:

1. Biologic assay for the sodium inhibitory factor in CF: Rat intestine was used to measure the mucosal-to-serosal flux of ^{24}Na (in the presence of saliva), the uptake of ^{14}C-3-0-methylglucose (in the presence of plasma) and the uptake of ^{14}C-alanine (in the presence of saliva). The effects of saliva on the influx and outflux of ^{24}Na in normal human erythrocytes were also measured. Labeled carbon was used as a volume marker in the appropriate situations. Methods employed were those previously described by Gardner, et al. and Agar, et al.

2. Jejunal immunoglobulin A (IgA) synthesis in CF: We have evaluated local IgA production by jejunal mucosa *in vitro* in 7 patients with CF, 7 patients with HP, and 29 normal subjects. IgA synthesis in biopsy specimens obtained perorally was assessed by measuring ^{14}C-L-leucine incorporation into IgA. Incorporated counts binding to specific anti-IgA antibodies covalently linked to bromacetyl cellulose constituted a measure of IgA production.

3. Human amniotic fluid (AF) isoamylase: Twenty-two AF samples were obtained at various times during gestation by amniocentesis or abortion and at delivery by trans-vaginal needle aspiration. Homogenized placenta, neonatal urine and serum, adult serum, duodenal fluid, and parotid saliva, and adult homogenized liver were also studied. Total AF amylase concentration was measured by an amyloclastic method. Disc polyacrylamide gel electrophoresis was used to separate the isoamylases and the various bands were identified by squeezing

the isoenzyme-containing polyacrylamide gel column against a starch agar film. The isoenzymes of amylase were allowed to react with the starch substrate: 15 min. at room temperature for saliva and duodenal fluid and 6 hrs. at 37°C in a moist box for urine, serum, placenta, liver, and AF. After incubation, the zymograms on the starch slides were developed in an iodine solution.

4. Tissue culture studies: Patient material consisted of known patients with cystic fibrosis, parents (obligate heterozygotes), and normal controls. Skin punch biopsies were obtained and cultured in Eagle's medium reinforced with 10% fetal calf serum. Fibroblasts were subcultured and either grown in quantity and harvested for biochemical studies or grown on glass coverslips for fixation and staining, or grown in flasks for subsequent harvesting and light microscopy. Glucose uptake experiments include timed experiments with 3-0-methylglucose to determine the rate of glucose uptake by the cell membrane. We are looking at comparative uptake and diffusion of this compound, the effect of insulin and fetal calf serum on glucose transport, energy requirements of the cell during the course of the experiments (i.e. Do varying rates of uptake merely reflect varying amounts of stored glycogen at t = 0?).

We are also engaged in cooperative studies with Dr. Alexander Spock to investigate the effect of fibroblast sonicates and media on ciliary beat by rabbit trachea; morphologic studies of fibroblasts by electron microscopy with Dr. Samuel Spicer; investigation of methylation of RNA by CF and normal fibroblasts with Dr. Owen Rennert.

5. Flow rates and electrolytes in minor salivary gland saliva in normal subjects and patients with CF: A new method for extracting saliva from small salivary glands from the inner aspect of the upper lip was devised. This method allows for the first time determination of sodium, potassium, calcium, and magnesium, as well as flow rates. Ten normal adults, 18 to 20 years of age, and 15 patients with CF, 11 to 28 years old, were included in this study.

Major Findings:

1. Effects of saliva and plasma from CF patients on membrane transport *in vitro*: We have investigated the effects of mixed saliva and plasma from CF patients on transport of sodium, alanine, and sugar in patients with CF of both sexes, 12 to 28 years of age, and controls of both sexes, age 18 to 32 years. There were no significant effects of saliva or plasma from patients with CF on any of the transport phenomena studied.

Additional preliminary studies also indicate that CF saliva, fibroblast homogenates, and tissue culture media from fibroblast cultures do not affect resting transmembrane potentials or short-circuit current across frog skin membranes *in vitro*.

2. Jejunal immunoglobulin A (IgA) synthesis in CF: Immunologic abnormalities have recently been described in patients with CF. The patients with CF were 14.5 to 41 years of age and their Shwachman scores ranged from 52 to 88.

The patients with HP were 14 to 44 years of age. Pancreatic enzymes were absent in all CF patients and in 2 of 7 HP patients; in the remaining 5 HP patients enzyme levels were significantly reduced to 20% of normal.

The mean IgA incorporation value in CF patients was 20373 ± 9244 cpm/mg protein (mean ± SD), and in HP patients was 11403 ± 4585, both values significantly greater than normal (6688 ± 2449) ($p < 0.001$). In addition, the mean IgA incorporation value in CF patients was greater than the value in HP patients ($p < 0.05$). Three of 7 biopsies from CF patients revealed increased numbers of plasma cells on histological examination. There was no correlation between IgA incorporation values and serum IgA levels, age, Shwachman score, severity of lung disease, type of bacteria in sputum cultures or roentgenographic abnormalities of the small bowel.

3. Human amniotic fluid (AF) isoamylase: An investigation of the amylase concentration and isoenzyme pattern in AF was performed since the source of the AF amylase has been in doubt. Amylase isoenzyme patterns in human AF have not been examined previously. The concentration of AF amylase rises significantly during gestation and may be as high as 248 Somogyi units/100 ml at term. The AF amylase isoenzyme pattern was nearly identical to that of neonatal urine and revealed distinct salivary (at least 5 bands) and pancreatic (at least 1 band) components. Isoamylase patterns from homogenized placenta were similar to that of adult serum and different from AF. The amount of amylase in placenta is low and suggests that it is of maternal origin. Maternal proteins (except for IgG) are not known to cross the placenta in appreciable amounts. The similarity of the AF and neonatal urine patterns indicate fetal origin of AF amylase. In contrast to previous beliefs, it appears that the pancreas and salivary glands of the fetus function early in intrauterine life (as early as 16 weeks).

4. Tissue culture studies: Preliminary results disclose that CF fibroblasts utilize 3-0-methylglucose at a faster rate than normal cells. These results are consistent and seem not to be due to a "trapping" phenomenon (as tested by ^{14}C inulin) or to a simple difference in passive diffusion across the membrane (as tested by the ability of "cold" glucose to suppress uptake). Enzyme activity as related to glucose metabolism will also be investigated. All of the above experiments must be controlled for cell age as increased glycogen in CF fibroblasts is not seen prior to 10 days after inoculation. Further experiments will involve investigation of the relationship of glucose to sodium transport with an eye to relating the increased glycogen to the sodium transport abnormality of CF.

Results concerning the ciliary factor (data from Dr. Alexander Spock) indicate that although fibroblast sonicates and tissue culture media will sporadically disrupt ciliary beat, no significant differences were found between CF and normals. Preliminary results from Dr. Owen Rennert disclose that fibroblasts from CF homozygotes and heterozygotes incorporate methionine at a significantly slower rate than do normals.

5. Flow rates and electrolytes in minor salivary gland saliva in normal subjects and patients with CF: Sodium was found markedly elevated and flow rates moderately decreased in patients with CF, whereas the other ions determined were equal to those of normal controls. In both groups sodium increased with increasing flow rates, but conversely potassium, magnesium, and calcium decreased inversely to the rates of flow.

Significance to Biomedical Research:

1. The ability of CF plasma, sweat, or saliva to alter the transport of a particular substance *in vitro* offers the potential for wide-scale screening for CF detection of heterozygotes, and further characterization of the molecular nature of the constituent which produces the observed alterations. Although we could detect no differences in the four systems studied, further investigations should be performed.

2. The most reasonable explanation for the IgA findings is that patients with CF and HP are under an increased local antigenic stimulus, perhaps due to an altered gastrointestinal environment which leads to increased local IgA production. This environment does not appear to be due to increased bacterial stimulation and implies that non-pathogenic material may be able to stimulate the production of locally-produced IgA.

3. Knowing the time of appearance and origin of amniotic fluid amylase may allow for the detection of pancreatic deficiency in utero in cystic fibrosis. This may be of significance for the antenatal diagnosis of this disease. Additionally, knowing the prenatal course of CF may allow one to apply certain therapeutic procedures (in the future) antenatally in correcting basic lesions of this disease.

4. The findings in tissue culture in cystic fibrosis are important in several respects: 1) They represent the first available experimental model outside the patients' bodies; 2) it now appears possible that in reality all cells are involved in the process leading to the manifestations of this generalized disease, while in the past CF was felt to be limited to the exocrine glands; 3) detection of biochemical abnormalities in fibroblasts may pave the way for a test to detect the heterozygotic state and/or provide the means for a prenatal diagnosis.

5. It is notable that minor salivary glands are the only exocrine gland system in addition to the eccrine sweat glands that consistently manifest the sodium abnormality in CF. In addition, as shown by the normal values for calcium in the patients tested in this study, the sodium defect appears to be independent of the increased concentration of calcium, as recently postulated for sweat glands by other authors.

Honors and Awards: None

Publications:

di Sant'Agnese, P.A.: The basic defect of cystic fibrosis: What is it? International Congress of Pediatrics, Wien, Osterreich, 1971, pp. 531-535.

Lapey, A., and Gardner, J.D.: Abnormal erythrocyte sodium transport in cystic fibrosis of the pancreas. Pediat. Res. 5:446-451, 1971.

Taussig, L.M., and Gardner, J.D.: Effects of saliva and plasma from cystic fibrosis patients on membrane transport. Lancet (in press).

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Clinical Studies in Cystic Fibrosis.

Previous Serial No.: None

Principal Investigator: Paul A. di Sant'Agnese, M.D.

Other Investigators: L.M. Taussig, M.D., J. Kattwinkel, M.D., A.P. Simopoulos, M.D. (Guest Worker), I.R. Boyle, M.D. (Guest Worker), S. Agus, M.D., NIAMDD; R.M. Saldino, M.D., B.E. Statland, M.D., CC; W.T. Friedewald, M.D., J.I. Verter, M.S., F.C. Bartter, M.D., NHLI.

Cooperating Units: Digestive and Hereditary Diseases Branch; Diagnostic Radiology, Clinical Pathology, CC; Biometrics Research Branch, Endocrinology Branch, NHLI; C.C. Lobeck, M.D., Univ. of Wisconsin, Madison; Donald Ackerman, Ph.D., Univ. of California, Los Angeles; F. Murad, M.D., Univ. of Virginia, Charlottesville; C.D. Arnaud, M.D., Mayo Clinic, Rochester, Minn.; Frances Millican, M.D., Georgetown Univ. Hospital, Washington, D.C.; Sallyann Sack, M.Phil., George Washington Univ., Washington, D.C.

Man Years: Total: 3.75
Professional: 2.5
Other: 1.25

Project Description:

Objectives:

1. The prevailing opinion has been that males with cystic fibrosis are sterile due to aspermia secondary to abnormal mesonephric derivatives. The existence of fertile males with this disease would have obvious genetic, social, psychological, and therapeutic implications.

2. Although the roentgenographic picture of the obstructive intestinal complications of cystic fibrosis are well known, little has been written about the x-ray abnormalities of the duodenum and small bowel in this disease. Abdominal pain of obscure etiology is a relatively common symptom in CF, and a high incidence of duodenal ulcers has been reported for patients. Barium studies of the duodenum and upper gastrointestinal tract were undertaken to further study these clinical manifestations.

3. The improved life expectancy of patients with cystic fibrosis has been associated with an increased frequency of certain complications, some of which have considerable prognostic significance. Pulmonary parameters rather than nutritional and dietary aspects currently appear to be associated with prognosis. A need has arisen for a systematized scoring system based as much as possible on objective criteria; such a system could be used to prognosticate and as a uniform and consistent method of evaluating and following patients for clinical purposes and studies. Previous scoring systems have been more subjective and have not considered these newer complications.

4. It is well known that patients with cystic fibrosis may develop hepatic biliary cirrhosis which may lead to significant clinical problems during life. Serologic tests for liver function in children have been difficult to interpret because of the lack of good normal data in this age group. Alkaline phosphatase (AP) in particular is known to increase markedly during adolescence secondary to active bone growth, thus making interpretation of this test as a measure of liver function most difficult. We have examined the sera from normal children and CF patients in an attempt to: 1) establish new norms for serologic liver tests in children; 2) demonstrate the age-independency of the liver isoenzyme of alkaline phosphatase; 3) establish the incidence of serologic evidence of liver disease in CF.

5. It has been reported that orally administered L-arginine is beneficial in decreasing the amount of steatorrhea, abdominal pain, and improving weight gain in patients with cystic fibrosis. It also has been suggested that oral administration of bicarbonate ($NaHCO_3$) may improve the malabsorption associated with pancreatic insufficiency due to other causes. In view of these findings, a study was undertaken to investigate the efficacy of orally administered L-arginine and $NaHCO_3$ in the treatment of malabsorption due to CF.

6. Previous studies from this Branch have revealed that patients with cystic fibrosis failed to show the expected decrease of urinary phosphorus following calcium infusion - results consistent with a state of secondary or tertiary hyperparathyroidism. To further delineate the abnormality, the responsiveness of bone and kidney to parathyroid extract (PTE) and the rate of calcium absorption were studied.

7. As more patients with cystic fibrosis are reaching adulthood, it has become important to assess psychological as well as physical performance and to determine how these patients can best be helped to function well. Therefore, an in-depth psychiatric evaluation was made of adolescents and young adults with CF.

Methods Employed and Patient Material:

1. Fertility in males with CF: Semen specimens were obtained from two unrelated CF male patients, one of whom had fathered two children. Semen was studied for volume, pH, sperm count, sperm motility and morphology, and citric acid, acid phosphatase, and fructose concentrations by standard methods.

2. Roentgenographic abnormalities of the duodenum and small bowel in CF: Thirty-three patients with CF, age 4 to 24 years, and 7 with hereditary pancreatitis (HP) were examined with barium esophagogram, upper gastrointestinal series, and small bowel roentgenograms; in addition 4 patients with CF had hypotonic duodenography. Biopsies of the proximal jejunum were done on 10 CF patients and all HP patients. X-rays of the duodenum and small bowel were scored separately on a 0 to 4 scale, without knowledge of the patients' histories.

3. Prognostic score and clinical evaluation sheet for CF: A new, easily-used system for patients with CF was devised. Seventy-three patients and their x-rays were evaluated independently by two of us. The age range was 5 to 30 years; 40 were male, 33 female. Three and 6-year survival rates were calculated and correlated with the components of the score sheet to determine which factors could be related directly with prognosis.

4. Isoenzymes of alkaline phosphatase (AP) and related serologic liver function studies in normal adolescence and in CF: An automated technique has been developed requiring 10 microliters of serum to examine total AP and its l-phenylalanine and urea stable components. This technique has been utilized as well as conventional methods for measuring 4 other serological parameters of liver disease: transaminases (SGOT, SGPT), 5'nucleotidase (5'N), and γ-glutamyl transpeptidase (γGTP). The sera of 91 normal controls (age 4-30) and 49 patients with CF were examined.

5. Treatment of malabsorption - arginine and sodium bicarbonate: Ten CF patients, ranging in age from 11 to 24 years, and with stable pulmonary disease were studied. The absence of proteases in the duodenal fluid was confirmed by duodenal intubation within 18 months of the study. A 100 g fat diet was ordered and actual fat and nitrogen intake was computed daily by a dietitian. Stools were collected in 72-hour pools and 2 or 3 pools were obtained for each period. The study consisted of 4 phases in most of the patients: no supplement, arginine alone, Cotazym alone, Cotazym plus $NaHCO_3$.

6. Parathyroid function in patients with CF: Parathyroid extract (PTE) in graded doses of 400 to 600 units for 3 days each was given intramuscularly while 5 patients were receiving a diet of 400 mg calcium and 800 mg phosphorus which was low in collagen. Urinary cyclic adenine monophosphate (cyclic AMP) was measured by Dr. Ferid Murad before and after PTE.

Gastrointestinal absorption of calcium was studied by the measurement of forearm radioactivity in a large-volume liquid scintillation counter following separate oral and intravenous doses of $^{47}CaCl_2$.

7. Emotional adjustment of adolescents and young adults with CF: Twenty-seven patients, ages 13 to 30, had psychiatric interviews, intelligence tests, and projective psychological testing. Fourteen patients were adolescents, ages 13 to 18, and thirteen were young adults, ages 19 to 30. In 21 families the patient's mother was seen and interviewed. Evaluations were made

as to the patient's functioning at work and school, self-esteem, personal relationships, and coping with illness.

Major Findings:

1. Fertility in males with CF: Semen from Case #1 (age 26) revealed normal seminal volume, sperm count, sperm motility and morphology, pH, citric acid and fructose concentrations. Case #2's (age 24½) semen also had a normal sperm count and seminal volume; he fathered two children. Blood group analysis was consistent with paternity.

The sterility found in most CF patients is due to aspermia and a low volume of ejaculate, secondary to abnormal, atretic or absent epididymides, vasa deferentia, and seminal vesicles (all mesonephric derivatives). In aspermic CF patients semen is primarily derived from prostatic secretions as shown by its acidic pH, high citric acid and acid phosphatase content and conversely by the low or absent fructose. The normal urinary tracts in CF patients indicate that when infertility occurs, mesonephric development proceeds normally until the 10th or 12th week of gestation. Maldevelopment may occur at variable times thereafter in fetal life or even after birth and the variety and type of such findings on pathologic examination suggest it may be secondary to obstruction of structures by abnormal secretions.

2. Roentgenographic abnormalities of the duodenum and small bowel in CF: Eighty-four percent of the patients with CF had roentgenographic abnormalities of the duodenum, 61% with moderate to severe changes. The abnormalities consisted of markedly thickened folds, filling defects, mucosal smudging, and dilatation and redundancy of the duodenum. There was no correlation between the duodenal x-ray findings and age, sex, weight percentile, Shwachman score, severity of lung disease, abdominal pain, history of intussusception or volvulus, hypoalbuminemia, amount of supplemental pancreatic enzymes being taken, pancreatic insufficiency as determined by duodenal intubations (22 patients), or histological material obtained from biopsies and autopsies (2 patients). The etiology of duodenal changes in CF is obscure. All patients with HP had pancreatic deficiency as determined by duodenal intubations and 72-hour stool pools for fecal fat, but x-rays of their duodenums were normal (5 cases) or showed slightly thickened folds (2 cases).

Nine patients (27%) with CF had abnormalities of the jejunum consisting mostly of thickened or coarse folds. Only rarely were findings compatible with a "deficiency" pattern noted; dilatation and segmentation and flocculation of the barium were observed in 2 cases and prolonged transit time was evident in only one patient. Three patients with HP also had minor small bowel changes. No ulcers were noted.

3. Prognostic score and clinical evaluation sheet for CF: The system, as derived, contains 2 parts: 1) The Prognostic Score involves primarily pulmonary parameters and totals 100 points. Seventy-five of these are concerned with chest x-ray, pulmonary function tests (V.C. and FEV_1), pulmonary exacerbations, pneumothorax, hemoptysis, pulmonary surgery, cor pulmonale, physical examina-

tion of the lungs, clubbing, and cough and sputum production. The remaining 25 points involve nutritional status, activity, and patient attitude toward his disease. 2) The Clinical Evaluation Section does not affect prognostic index and considers other common complications and additional tests not easily obtained on routine clinic visits.

4. Isoenzymes of alkaline phosphatase (AP) and related serologic liver function studies in normal adolescence and in CF: Our results disclose that total AP is a poor indicator of minimal biliary cirrhosis in childhood because of the masking effect of bone isoenzyme. Determination of the liver fraction of AP, however, renders the test age-independent and unmasks a significant number of abnormal values. Thirty-five percent of the total AP results were represented by misleading values when compared to the liver isoenzyme and to the other serologic tests. Conclusions to be drawn are that: 1) measurement of total AP without regard to organ source of the enzyme ignores variations in bone growth and concomitantly variations in bone isoenzyme which in turn results in imprecise norms for the pediatric age group; 2) the extremely high levels of bone isoenzyme of AP during childhood tend to "mask" moderate increases secondary to elevated liver AP or other AP isoenzymes; 3) in normal children levels of SGOT, γGTP, total AP and its bone isoenzyme are age-dependent, whereas SGPT, 5'N, and intestinal and liver fractions of AP are independent of age up to 30 years; 4) comparison of age-matched data from normals with CF patients discloses that about 1/3 of CF patients have serologic evidence of hepatobiliary disease and that γGTP, the transaminases, and the liver isoenzyme of AP are the most reliable indicators of this disease.

5. Treatment of malabsorption - arginine and sodium bicarbonate: The results disclosed that in contrast to previous reports, in the patients studied, L-arginine produced no improvement in the amount of steatorrhea or azotorrhea. In addition, all patients on arginine lost body weight (mean = 1.0 kg) in contrast to the weight gain observed on all other regimens. Although 4 of 8 patients showed improvement in these parameters when $NaHCO_3$ was added to Cotazym alone, the mean improvement for all patients was not statistically significant.

6. Parathyroid function in patients with CF: Serum calcium increased, serum phosphorus decreased, urinary calcium and hydroxyproline increased, urinary phosphorus increased after PTE. In all patients urinary cyclic adenosine monophosphate was elevated during the control period, in three patients it increased even further with PTE and returned to control values after PTE was discontinued.

Gastrointestinal absorption of calcium was within the normal range for all subjects despite the presence of malabsorption.

The data indicate that the response to PTE administration in patients with CF does not differ from that of normal subjects, indicating a normal end organ responsiveness. However, the secretion of parathyroid hormone does not suppress normally and is independent of the control of serum calcium.

7. Emotional adjustment of adolescents and young adults with CF: Psychologically, patients with CF were found to be under considerable stress. Aside from anxieties about their illness, difficulties in their upbringing contributed to emotional instability. Sixty percent of the patients' fathers were perceived as distant and unsupporting. Of the patients who saw their fathers in this manner, 65% showed lower self-esteem and made fewer active attempts to master the external world. In terms of self-image, patients from families which openly discussed CF were more likely to see themselves as different from others, but were more accepting of this difference. Those from families with no communication about CF denied differences, describing themselves as the same as other people. However, their projective tests showed more anxiety, inferiority, and anger.

These patients were found to have compensated for physical defects in terms of achievement. However, infantilizing relationships with mothers, withdrawal of the fathers, and denial of the emotional impact of the illness contributed to psychological difficulties in adult life. The question of psychiatric support for these patients and their families needs to be investigated further. Possible considerations are group therapy for parents and for spouses of CF patients.

Significance to Biomedical Research:

1. Fertility in males with CF: Fertility occurs in a small but significant percentage of male patients with CF and it is mandatory to evaluate the semen from all post-pubescent males with CF prior to counseling. Males with CF can no longer be considered as sterile without appropriate evaluation. These findings have obvious genetic and psychological implications.

2. Roentgenographic abnormalities of the duodenum and small bowel in CF: It is important to recognize that x-ray abnormalities of the duodenum may occur in CF so that faulty diagnoses are not made; other distressful and expensive diagnostic procedures thus can be avoided. The relationship of these findings to the abdominal pain of obscure etiology which commonly occurs in CF is still unclear. No changes of acute or healed duodenal ulcers were observed, suggesting that ulcers noted at autopsy in CF may be terminal events.

3. Prognostic score and clinical evaluation sheet for CF: We feel that this system supplies information not provided by existing methods. It allows for registering and appropriately weighing various parameters in a more objective manner and provides the clinician with a concise summary of an individual patient's clinical course and complications. The physician newly introduced to CF may quickly assess in a systematized manner a patient's history, course, and prognosis. Finally, it allows for easy categorization of patients for accurate patient comparison for research purposes.

4. Isoenzymes of alkaline phosphatase and related serologic liver function studies in normal adolescence and in CF: These results are helpful in interpreting serologic enzyme results in children and disclose that serologic

evidence of liver disease in CF may be detected early before clinical signs are evident, thus providing the means for accurate follow-up and more reliable prognosis of this complication.

5. Treatment of malabsorption - arginine and sodium bicarbonate: The oral use of L-arginine is of no benefit in the treatment of malabsorption due to CF. Although $NaHCO_3$ may be of benefit in certain selected cases, it should not be a part of the routine treatment of malabsorption due to CF.

6. Parathyroid function in patients with CF: As the basic defect in CF is not yet known, it is of obvious importance to define further the endocrinologic status in these patients. A defect of calcium metabolism appears to be present. Its clinical implications and relationship to abnormal calcium binding, previously found to be present in secretions from CF patients, remains to be determined.

7. Emotional adjustment of adolescents and young adults with CF: With the improved life expectancy for CF, psychological problems associated with adolescence and young adulthood will continue to confront the physician responsible for the care of the CF patient. Further knowledge of the pathophysiology of psychiatric disease associated with CF thus will be essential in the comprehensive treatment of cystic fibrosis.

Honors and Awards: None

Publications:

Kattwinkel, J., Agus, S.G., Taussig, L.M., di Sant'Agnese, P.A., and Laster, L.: The use of L-arginine and sodium bicarbonate in the treatment of malabsorption due to cystic fibrosis. Pediatrics (in press).

Taussig, L.M., Lobeck, C.C., di Sant'Agnese, P.A., Ackerman, D.R., and Kattwinkel, J.: Fertility in males with cystic fibrosis. New Eng. J. Med. (in press).

di Sant'Agnese, P.A.: Malabsorption syndromes (Celiac syndrome). In Gellis, S.S., and Kagan, B.M. (Eds.): Current Pediatric Therapy (ed. 5). Philadelphia, W.B. Saunders Co., 1971, pp. 242-249.

Simopoulos, A.P., Lapey, A., Boat, T.F., di Sant'Agnese, P.A., and Bartter, F.C.: The renin-angiotensin-aldosterone system in patients with cystic fibrosis of the pancreas. Pediat. Res. 5:626-632, 1971.

Serial No. NIAMDD-PMB-3c
1. Pediatric Metabolism
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies in Familial Inherited Pancreatitis.

Previous Serial No.: Same

Principal Investigator: Paul A. di Sant'Agnese, M.D.

Other Investigators: John Kattwinkel, M.D., J. Charles Pallavicini, Ph.D., W. A. Edwards, B.S., NIAMDD.

Cooperating Units: Digestive and Hereditary Diseases Branch, NIAMDD.

Man Years: Total: 1.5
Professional: 1.0
Other: .5

Project Description:

Objectives:

Three large kindreds with hereditary pancreatitis are being studied in order to define the inheritance pattern, incidence of complications (e.g. pancreatic insufficiency, pancreatic calcification, diabetes, abdominal carcinoma), pathogenetic mechanisms, and best forms of therapy for this relatively new genetic pancreatic disease.

Methods Employed and Patient Material:

We have studied three large kindreds located in a small area of Tennessee, West Virginia, and Virginia respectively. Although the families reside within an area of less than 200 miles in diameter, we have been unable to establish definite inter-family relationships. With 30 definite cases of pancreatitis, the present kindreds represent more than one-third of the total previously described cases. In the families under study other metabolic defects have been excluded by detailed studies of pancreatic and parathyroid function, serum lipids, pathologic specimens, and urinary amino acid content.

Major Findings:

HP, although similar in many ways to the adult picture, has many distinctive features: lack of sex incidence, onset usually in early childhood, rarity of alcoholism and gall stones, early onset of calcifications, relative rarity of diabetes mellitus, and good life expectancy.

It is evident, therefore, that at least in the middle eastern section of the United States, hereditary pancreatitis is not a rare disease and usually presents in the childhood age group. HP, indeed, is the most common cause of relapsing pancreatitis in childhood and should always be considered in the differential diagnosis of recurrent abdominal pain and abdominal calcification in the pediatric age group. Contrary to classical teaching, the existence of chronic inflammation of the pancreas with calcification appears to predispose to resultant pancreatic malignancy late in adult life.

While the etiology and pathogenesis of HP are still a mystery, the autosomal dominant type of transmission suggests that the basic defect is not an inborn error of metabolism, but a structural defect. The aminoaciduria reported in two of the five original HP kindreds was probably due to coexistence of HP in incompletely recessive cystinuria.

Significance to Biomedical Research:

The further definition of the genetic transmission, clinical and laboratory manifestations, and of the prognostic implications of this relatively new disease will have important implications in pediatric and adult gastroenterology. It will further our knowledge of the relatively unknown and new group of pancreatic disorders of genetic origin.

Honors and Awards: None

Publications:

Kattwinkel, J., Lapey, A., di Sant'Agnese, P.A., and Edwards, W.A.: Hereditary Pancreatitis: Three New Kindreds and a Critical Review of the Literature. Pediatrics (In press).

I. Study of Blood Coagulation and Diseases of Hemmorrhage and Thrombosis

1. The platelet release reaction

Thrombin, collagen, epinephrine and certain polycations release ADP from platelets and also aggregate platelets. Many workers have suggested that release of ADP in the final common pathway for aggregation of platelets by these diverse agents. In attempts to define the minimal conditions necessary to produce aggregation of platelets in a saline medium we found that each of the different aggregating agents had specific ionic and plasma protein requirements and that release of ADP and aggregation of platelets were separable phenomena. ADP, therefore, does not appear to be the agent responsible for aggregation. The specific ionic requirements of collagen aggregation suggest that a previously postulated glucosyl transferase reaction may be involved.

2. Utilization of the platelet release reaction to measure ITP factor and platelet antibodies

Many anti-platelet isoantibodies and the anti-platelet factor responsible for ITP cannot be measured by conventional immunologic techniques. However, antibodies, like various other agents that affect platelet membranes, release intracellular substances, some of which can be measured in trace amounts. In the present work, methods were developed for detecting release of C^{14}-labeled serotonin and ATP from platelets injured by levels of antibody and types of antibodies not previously detectable by immunologic techniques. The methods are useful for diagnosing isosensitization clinically and for detecting ITP factor.

3. Mechanism of formation of thrombin from prothrombin

Continued studies of the kinetics of conversion of prothrombin to thrombin in a purified coagulation system has lead to further definition of an intermediate compound between prothrombin and thrombin. The intermediate was found to have fibrinogen-clotting activity indistinguishable from thrombin but the intermediate with thrombin activity combined with the proteolytic inhibitor, soybean trypsin inhibitor, whereas the final stable thrombin did not combine with proteolytic inhibitors. The kinetics of this system is helpful in explaining the homeostasis of blood coagulation.

4. Purification and characterization of Factor VIII (antihemophilic globulin

Factor VIII (antihemophiliac globulin) was purified by Sepharose filtration after unique treatment of crude starting material with proteolytic enzymes to eliminate the major contaminant, fibrinogen, that has interfered with most other methods of purification. The purified protein was analyzed chemically with respect to carbohydrate, lipid and amino acid content. It is a glycoprotein with a major subunit of 240,000 that self polymerizes to form aggregates greater than one million in molecular weight.

5. Effects of platelet aggregating agents on soluble proteins from platelets

Attempts were made to determine whether the various agents that cause platelet aggregation react with soluble proteins that can be extracted from platelets. The only aggregating agent that changed soluble proteins was thrombin which produced the same changes in platelet fibrinogen that it produces in plasma fibrinogen.

II. Study of the Immunology of Blood Cell Deficiencies

1. Antibodies arising during hepatitis that react with antigens in normal stools

In attempts to identify the antigen associated with viral hepatitis B in stools of patients with hepatitis, an antigen was discovered that reacts with sera from patients with actue hepatitis. This antigen, however, differed from hepatitis B antigen and from all other antigens known to react with naturally occurring human antibodies. It appeared that there is an antigen in stools of normal individuals that gains access to antibody forming tissue during acute liver disease by virtue of impaired hepatic filtration function. The antigen appears to be bacterial or dietary in origin. Reactions of this antigen with antibodies arising during hepatitis could play a role in the immunologic manifestations of acute hepatitis that resemble antigen-antibody complex disease.

2. A test for antibodies against toxoplasma

Using a technique that had been developed to measure antibodies against hepatitis antigen, a test for antibodies against toxoplasma organism was developed. The current tests that are available for toxoplasma antibodies depend on complex equipment, expensive and labile reagents and skill of the technician for interpretation. The hemagglutination method that we developed is simple, stable and can be carried out by those without special laboratory training. Because of the potential large scale commerical application of this technique, particularly in obstetrical clinics because one in one thousand infants have toxoplasmosis, a patent has been submittied.

3. Occurrence of hepatitis-associated antigen and antibody in excreta and secretions

In the course of evaluating the epidemiology of hepatitis B, we attempted to determine whether the hepatitis B antigen which is present in infectious plasma is also in materials such as saliva, stool and urine which are likely sources of transmission of a viral agent by the non-parental route. While some investigators have reported finding this antigen in various body excreta and secretions we were unable to do so.

4. Antibody response in viral hepatitis, type B

Using a sensitive technique to measure antibodies against the hepatitis type B antigen we have for the first time been able to detect antibodies in a high percentage of patients recovering from acute viral hepatitis. Sera from 88 cases of viral hepatitis were examined during the incubation, acute and convalescent periods and 79.5% were found to develop antibodies against hepatitis B antigen at sometime during the course of the disease. Of those who developed antibody 75% had what appeared to be primary antibody responses late in the course of the disease whereas 24.3% had apparent secondary antibody responses earlier in the course of disease. Most individuals who were resistant to infection after parental exposure had secondary antibody responses. Protective immunity to infection with hepatitis B virus was not assured by previous exposure to that agent for 24% of patients who developed hepatitis had secondary antibody responses. Since none of the individuals had prior history of transfusion or hepatitis it appears that exposure in the general population by means other than transfusion is a common event.

5. The serology of post transfusion purpura

We further studied the serology of post-transfusion purpura in an unusually severe and fatal case. Although most antibodies that arise in this disease are complement fixing and specific for a single platelet antigen, $P1^{A1}$, this patient developed an antibody which fixed complement with most, but not all $P1^{A1}$-positive platelets. On further study it was found that occasional platelets are present in normal individuals that react with the antiserum to absorb antibody but not fix complement. Further study of the unusual antiserum and unusual platelet promises to provide information concerning mechanisms of complement fixation.

6. Histochemical studies of megakaryocytes and platelets

Histochemical techniques have been developed to provide semiquantitative assays for acid phosphatase, cytochrome oxidase and glycogen in megakaryocytes and platelets. Current work suggests that levels and distribution of these substances may be correlated with the age and function of these cells.

Serial No. NIAMD-CHB-1c
1. Clinical Hematology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Study of Blood Coagulation and Diseases of Hemmorrhage and Thrombosis

Previous Serial Number: SAME

Principal Investigator: Dr. N. Raphael Shulman

Other Investigators: Dr. Richard Hirschman
Dr. Roger Lange
Dr. Peter Tomasulo

Cooperating Units: Dr. Sally Marchesi, CC, CP
Dr. Harvey Gralnick, CC, CP
Dr. Wayne London, MR, NIAMD
Dr. John Hearon, MR, NIAMD
Robert Morton, Oklahoma Medical School

Man Years
Total: 3.5
Professional: 2.0
Other: 1.5

Project Description:

Objectives:

Study of the reactions and interactions of coagulation factors in vitro and in vivo to define further the nature of the blood coagulation mechanism, to determine factors of significance in the pathogenesis of diseases of hemorrhage and thrombosis, and to develop better forms of therapy for these diseases.

Methods Employed:

Methods of protein purification and characterization, techniques of enzymology applied primarily to proteolytic enzymes and their inhibitors, kinetic analyses of enzyme reactions, procedures for quantitative measurement of various clotting factors, pharmacologic and physiologic techniques applied in man and animals, and assessment of metabolic pathways of blood cells with radioactively labeled substrates.

Major Findings:

1. The platelet release reaction

A number of unrelated substances including thrombin, collagen, epinephrine, ADP, and poly-L-lysine induce platelet aggregation. Many workers have found that these various agents also cause the release of ADP from platelets and it has been proposed that, with the exception of polycations, these various agents bring about platelet aggregation through the release of ADP and the subsequent direct effect of ADP on platelets. However, the effects of ADP and the effects of various factors in plasma on aggregation have not been clearly separated. The present work was undertaken to determine the minimal conditions necessary to produce aggregation of human platelets by various substances in a saline medium. Our findings indicate that ADP is not the final common pathway of thrombin or collagen-induced aggregation. In saline suspensions, platelets are not aggregated by substances that release ADP from platelets or by ADP added to the suspension. As reported by others, we found that a plasma factor was necessary but that this plasma factor was not fibrinogen as suspected by some workers. Heated plasma absorbed with aluminum hydroxide to remove or inactivate most known clotting factors did support platelet aggregation by ADP. The factor was not serum albumin as proposed by other workers for crystalline human albumin did not support aggregation whereas bovine albumin used by other workers may well have been contaminated by other proteins in trace amounts. It was found that Mg^{++} and Mn^{++} ions were more effective than Ca^{++} in supporting collagen induced aggregation. The results are consistent with the possibility that collagen-induced aggregation involves an enzymatic reaction such as glucosyl transferase which requires Mg^{++} or Mn^{++} and has recently been found to bind collagen to platelet membranes. It was further found that acetyl salicylic acid, sulfhydryl inhibitors which inhibit the glucosyl transferase of platelet membranes and strontium also inhibit platelet aggregation by collagen. None of these inhibitors, however, prevents release of ADP from platelets. Thrombin appears to react with platelets in the absence of extraneous fibrinogen possibly by affecting platelet fibrinogen for proteolytic enzyme treatment of platelets prevents platelet aggregation by thrombin. The divalent cation requirement for thrombin aggregation does not distinguish between Ca^{++}, Mg^{++}, or Mn^{++}, but is inhibited by strontium just like collagen aggregation. Lanthanum ion and poly-L-lysine cause aggregation of washed platelets directly without causing platelets to release nucleotides, possibly by binding to carboxylate groups of saliac acid in the membranes as in the case of red cell aggregation by polylysine and La^{+++}. Whereas thrombin, collagen and ADP stimulate the release reaction and increase platelet metabolism as measured by lactate and CO_2 production and incorporation of C^{14}-acetate into platelet lipids, La^{+++} and polylysine do not have these effects on platelets. These various studies indicate that the binding of thrombin, collagen, polycations and La^{+++} to platelets differs from that of ADP yet all of these substances result in platelet aggregation. Thus the binding of various substances of both low and high molecular weight to platelet membranes appear to take place by different and unique mechanism some of which can induce the release reaction and/or aggregation others either reaction independently. It is clear, however,

that there is not one common final pathway accounting for the platelet release reaction or subsequent platelet aggregation. (Richard Counts, Peter Tomasulo, Carla Knepp, Ceceil Coleman, N. Raphael Shulman)

2. Utilization of the platelet release reaction to measure ITP factor and platelet antibodies

Many anti-platelet isoantibodies responsible for in vivo platelet destruction can be measured by either complement fixation or platelet agglutination techniques. Some isoantibodies, however, do not react in these conventional immunologic tests. The anti-platelet factor found in the plasma of some patients with Idiopathic Thrombocytopenic Purpura also cannot be detected in vitro by any conventional immunologic technique. Investigators have therefore sought techniques that depend on platelet injury as indirect evidence for the presence of antibody. These techniques have involved the elution or release of various substances from platelets as, for example, chromium 51, histamine, adenine nucleotides or serotonin. The most recent tests involves acceleration of blood coagulation by "platelet factor 3" which apparently is made more available as a result of antibody attachment to platelets. All of these indirect tests, however, have either been too cumbersome for clinical application, not sensitive enough to detect ITP factor or frequently positive with control samples. In the present work two methods were developed for detecting many obscure anti-platelet antibodies and ITP factor by their ability to cause release of C^{14}-labeled serotonin and ATP from platelets. Using citrated plasma, 24 of 36 ITP patient plasmas caused significantly increased release of C^{14}-labeled serotonin from human platelets and a concomitant release of ATP measured in the firefly latern extract assay. The serotonin release method requires 2 hours whereas the firefly method requires only 30 minutes. Using release as an indicator, the ITP factor was found to be in the 7S gamma globulin peak on DEAE chromatography and it could be recovered from platelets by acid elution. The methods described herein are useful for diagnosing ITP and studying ITP factor as well as platelet isoimmunization and cross matching isosensitized recipients. (Richard Hirschman, Peter Tomasulo, Roger Lange, Ceceil Coleman, Cynthia Hicks, N. Raphael Shulman)

3. Mechanism of formation of thrombin from prothrombin

Continued studies of the kinetics of the conversion of prothrombin to thrombin in a purified coagulation system has provided evidence for an intermediate compound between prothrombin and thrombin. This substance was first identified in collaboration with the Mathematical Research Branch as the compound that combined with soybean trypsin inhibitor when this proteolytic inhibitor was used to prevent thrombin formation. In subsequent further studies of the mechanism of inhibition of prothrombin conversion by SBTI using the highly purified converting factor Xa the intermediate derivative of prothrombin was bound to have fibrinogen clotting activity indistinguishable from thrombin. The intermediate with thrombin activity could be further converted to a more stable compound (thrombin) that is unable to combine with proteolytic inhibitors. The marked rate-dependence of the kinetic system that had previously been recognized when crude biological factors were used to convert prothrombin was substantiated in the system of purified factors.

This provides an explanation for hemostasis of blood coagulation in that slow rates of prothrombin conversion are markedly inhibited by proteolytic inhibitors in plasma whereas rapid rates of prothrombin that would tend to occur extravascularly as a result of injury would not be inhibited significantly. (Wayne London, Richard Counts, Carla Knepp, N. Raphael Shulman)

4. Purification and characterization of Factor VIII (antihemophilic globulin)

One of the major problems in purification of Factor VIII has been separation of fibrinogen from other high molecular weight components of plasma. We have found that limited digestion by proteolytic enzymes of concentrates from plasma that are rich in both Factor VIII and fibrinogen resulted in formation of large non-coagulable fragments of fibrinogen and no loss in activity of Factor VIII. Factor VIII could then be purified by Sephrose gel filtration without contamination by native fibrinogen. The purified Factor VIII was found to be a macromolecular glycoprotein with a major subunit of 240,000 as shown by SDS-polyacrylamide gel electrophoresis. Carbohydrate analysis of Factor VIII gave values of 1% salicylic acid, 2.8% hexosamine, and 1-2% hexos (manos, lactos, and frucose). The lipid content was found to be less than 5% of the protein content and there was no detectable phospholipid. Immunoelectrophoretic analysis using rabbit antibody to purified Factor VIII produced a single precipitin line. There are several reports in the literature on purified Factor VIII that suggest that it contains phospholipid but it appears that this component is present only in Factor VIII preparations made from blood obtained from non-fasting individuals. Characteristic of our Factor VIII preparation, although made from human blood, are very similar to those of a purified Factor VIII preparation made from bovine blood by an elaborate precipitation technique described by Davie and co-workers. The amino acid content of bovine and human Factor VIII are very similar as are the carbohydrate and lipid moieties. Although Factor VIII appears to be over one million in molecular weight, subunits appear on SDS gels of approximately 240,000 MW. It is possible that Factor VIII has a specific molecular interaction with fibrinogen in plasma and may aggregate or polymerize when separated from fibrinogen. Interaction with fibrinogen may be important in maintaining a specific conformation of the Factor VIII molecule necessary for function for removable of fibrinogen by purification results in marked loss of clotting activity. (Sally Marchesi, Harvey Gralnick, N. Raphael Shulman)

5. Effects of platelet aggregating agents on soluble proteins from platelets

Membrane-free platelet extracts contain a variety of soluble proteins like extracts of other cells; but in addition, contain large amounts of high molecular weight contractile protein known as thrombosthenin and a thrombin-clottable protein known as platelet fibrinogen. The purpose of the study was to determine possible qualitative changes in soluble proteins, particularly the macromolecular components when exposed to aggregating agents such as thrombin, collagen, ADP, and epinephrine. Extracts of platelet proteins were first purified by elution from DE-23 columns. The

various aggregating agents were added to separate aliquots of reconstituted lyophilized peaks and the treated solutions were compared with untreated aliquots by acrylamide gel electrophoresis after solubilization of proteins in 10 M urea and 1% SDS-BME. Although thrombin produced changes in platelet fibrinogen that were comparable to changes in plasma fibrinogen, i.e. formation of gamma-gamma dimers and alpha polymers, there was no apparent effect of the other aggregating agents on the fibrinogen component of platelets or on thrombosthenin. The current work suggests that a fibrinogen-thrombin interaction may occur when platelets aggregate but does not substantiate a similar mechanism for platelet aggregation by other agents. (Robert Morton, Peter Tomasulo, N. Raphael Shulman)

Serial No. NIAMD-CHB-2c
1. Clinical Hematology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Study of the Immunology of Blood Cell Deficiencies

Previous Serial Number: SAME

Principal Investigator: Dr. N. Raphael Shulman

Other Investigators: Dr. Richard Hirschman
Dr. Roger Lange
Dr. Peter Tomasulo
Dr. Olivera Markovic

Cooperating Units: Dr. Lewellys F. Barker, LVI, DBS
Dr. Gerald Klatskin, Yale University
Dr. John Gockerman, Walter Reed Institute for Medical Research
Dr. Alfred Steinberg, ARB, NIAMD

Man Years

Total:	4.5
Professional:	3.0
Other:	1.5

Project Description:

Objectives:

To study the nature of immunologic reactions which result in formation of antibodies against autologous blood cells, and to determine the significance of immunity in development of "idiopathic" blood cell deficiency states. Of special interest are the biochemical reactions which result in formation of complexes between cells, antibodies, and drug haptenes and the physiologic processes which result in sequestration of cells with attached antibodies. In recent years our work on identifying inherited leukocyte and platelet isoantigens has led to a study of the significance of these antigens in hetero- and homo-transplantation and in possibilities of developing immunologic therapy against malignancies. Immunologic techniques, have been used to develop diagnostic techniques for evaluating the infectious diseases, hepatitis B and toxoplasmosis.

Methods Employed:

Techniques of quantitative immunochemistry, including preparation and physicochemical characterization of purified antibodies and antigens, micro-analyses for nitrogen, histamine, and alkaloid drugs, quantitative measurements of complement fixation, cellular agglutination and precipitin reactions, immuno-electrophoresis, methods of provoking antibody responses in man and animals, and isotopic and fluorescent labeling applied to antigens and antibodies. Methods of assessing reticuloendothelial function through clearance of labeled particles and organ localization of sequestered cells by external body counting. Tissue culture techniques, red cell agglutination techniques and lymphocyte transformation tests, including radioautography. Methods of hemagglutination involving attachment of various antigens to erythrocytes by chemical coupling.

Major Findings:

1. Antibodies arising during hepatitis that react with antigens in normal stools

In attempts to identify the antigen associated with viral hepatitis type B in stools of patients with hepatitis, stool extracts were reacted with sera from patients convalescening from hepatitis. A number of these sera formed a precipitin line with stool extracts but the stool antigen was immunologically distinct from the hepatitis B antigen (HBAg) as indicated by crossing a precipitin line in agar gel diffusion. Antibodies against the stool antigen appeared with highest frequency in patients with acute hepatitis whether HBAg-positive or -negative and also in multiply-transfused patients. The antibodies characteristically were 7S gamma globulin, did not fix complement and were of low avidity, forming reversible complexes that were soluble in saline. The stool antigen(s) differs from substances that have been described as reacting with natural occurring human antibodies. The antigen is present as frequently in normal stools as in stools of patients with hepatitis but is not found in urine, bile, serum, or extracts of liver, pancreas, stomach or various other organs including the intestinal tract. The molecular weight of the antigen varies from 60,000 to greater than 300,000 and appears to be protein in that it is digestible by trypsin and degraded by detergents. Since the stool antigen(s) is recognized by an antibody that develops during acute hepatitis or occasionally during severe bowel disease, it appears that during acute hepatitis a number of substances from the intestine, which are ordinarily filtered from the portal blood by Kupfer cells or parenchymal cells of the liver are not effectively removed and gain access to antibody forming tissue. Although not fully characterized as yet these antigens could be bacterial or dietary in origin. HBAg-anti-HBAg complexes have recently received much attention as possibly accounting for certain manifestations of hepatitis that might be caused by immune complexes. The antigen system above also could play a role in immunologic manifestations of acute hepatitis and other forms of liver disease. (Roger Lange, Carla Knepp, Ceceil Coleman, N. Raphael Shulman)

2. A test for antibodies against toxoplasma

Since one in one thousand newborn babies are infected with toxoplasmosis, antitoxoplasma tests are routine in most nurseries and obstetrical clinics. The current tests available for toxoplasma antibodies depend on fluorescent microscopy that requires skill as well as expensive and labile reagents or on a hemagglutination test that requires attaching toxoplasma antigens to sheep cells with tannic acid as a coupling agent. The labeled cells must be produced daily and human serum must be absorbed to prevent non-specific agglutination with sheep cells. In view of our experience with coupling the hepatitis associated antigen to red cells and developing a sensitive and simple clinical test for antibodies against the hepatitis antigen we used similar techniques to attach toxoplasma antigens to red cells in development of a stable hemagglutination test. Although various chemical procedures were used in attempts to attach toxoplasma antigen to inert carriers, the techniques that proved best involved the use of human erythrocytes stablized by aldehydes. After treatment with pyruvic aldehyde, erythrocytes are capable of coupling with toxoplasma antigen to form a stable and inert complex. The labeled cells after such treatment can be stored in the frozen or lyophilized state and used in a typical hemagglutination reaction after many weeks or months. The reagents are inexpensive, easy to use, and the end point of the reaction can be read macroscopically by individuals with no special training. Because of potential large scale commerical application a patent has been submitted. (Ceceil Coleman and N. Raphael Shulman)

3. Occurrence of hepatitis-associated antigen and antibody in excreta and secretions

There is evidence from the epidemiology of hepatitis B that this disease can be transmitted non-parentally but the only direct experimental evidence bearing on the oral transmission of hepatitis B is a single study in which infectious plasma fed orally transmitted this form of hepatitis. There is no other direct evidence that body fluids or excretment can transmit hepatitis B. We have attempted to determine whether the antigen associated with hepatitis B (HBAg), which is present in infectious plasma, is also in materials such as saliva stool and urine which are likely sources of transmission of a viral agent. While some investigators have reported finding HBAg in stools, urine, or bile we have not been able to confirm these observations. During our attempts to demonstrate HBAg in stools of patients with hepatitis B we discovered a new antigen system that appears unrelated to HBAg (see number 1 above). (Carla Knepp, Ceceil Coleman, N. Raphael Shulman)

4. Antibody responses in viral hepatitis, type B

Most individuals with viral hepatitis, type B, acquire a serum antigen (HBAg) at sometime during the incubation period or the acute disease. In the present study serial sera from 88 cases of viral hepatitis, type B, were examined for antibodies against HBAg by sensitive passive

hemagglutination and radioimmunoassay methods. In contrast to failure to detect antibody by previous techniques, 70 individuals (79.5%) were found to develop serum anti-HBAg (HBAb) sometime post-exposure by the hemagglutination and radioimmunoassay methods. Both methods gave the same results. Overt hepatitis cases were usually followed by primary type HBAg responses indicated by the detection of antibody shortly after HBAg disappeared during the acute phase or in the early convalescence stage of clinical hepatitis. In 9 cases, however, HBAb did not become detectable until 1 to 4 months after the disappearance of HBAg and often many weeks after clinical evidence of hepatitis had subsided. These types of late HBAb-responses were considered primary antibody responses and were observed in 53 of the 70 cases (75.7%) who acquired detectable antibody. In 17 of 70 cases (24.3%) who acquired serum antibody, the antibody was present between 1 and 4 weeks after inoculation of infectious material and prior to the appearance of HBAg in the serum or evidence of clinical hepatitis. These early antibody responses were considered secondary or an amnestic type responses reflecting prior exposure to HBAg. Early or secondary type HBAb responses were seen in 17 individuals who subsequently developed clinical hepatitis. Most individuals who were resistant to infection after parental exposure to materials known to transmit hepatitis had secondary antibody responses. It was concluded that primary HBAb responses are common after illnesses resulting from initial exposure to hepatitis B virus and that protective immunity to infection with hepatitis B virus frequently, but not invariably, follows early exposure to that agent. Our finding that antibody may fail to protect against reinfection may reflect the presence of relatively large amounts of infectious virus in the challenge materials used in the present study. It is possible, however, that the antibody against HBAg that is measured by currently avaiable techniques is not a neutralizing antibody, but rather an antibody against an outer "coat" antigen which is coded for, but antigenically distinguishable from the infectious virion core. None of the individuals in our study had a prior history of transfusion so that the route of previous exposure to HBAg in those who had secondary antibody responses is somewhat obscure. Exposure in the general population by means of other than transfusion appears from this study and from recent antibody studies of other investigators to be a common event. (Lewellys Barker and N. R. Shulman)

5. The serology of post transfusion purpura

In 1959 we described a syndrome of thrombocytopenic purpura occurring one week after transfusion as a result of isosensitization to a platelet antigen, $P1^{A1}$. Since that time we and others have seen a total of 20 cases of post transfusion purpura all involving sensitization to $P1^{A1}$. During the past year a patient was observed to develop fulminant purpura one week after a transfusion that was given during the course of a posterior fossa exploration for removal of a cerebellar tumor. The patient's serum, as in all other cases of post transfusion purpura, contained an anti-platelet antibody identified with the usual of $P1^{A1}$-positive and -negative platelets. However, one platelet preparation that was phenotypically $P1^{A1}$-positive by reaction with sera from several other cases of post transfusion purpura (PTP) did not react in complement fixation with the serum from the patient studied. In screening 15 sera from patients with PTP it was found that another patient whose serum fixed complement with all other $P1^{A1}$-positive

platelets on the panel did not fix complement with the unusual $P1^{A1}$-positive platelet. The antibody from both parents, however, was absorbed by the platelet which appeared to be non-reactive by complement fixation; and antibody eluted from the platelet preparation, did fixed complement with all other $P1^{A1}$-positive platelets on the panel. This unusual serologic finding suggested that an occasional patient may develop antibodies with more than one type of anti-$P1^{A1}$ activity, some of which are complement fixing and some of which are not. Certain platelets from normal individuals apparently have a distribution of antigen on their surfaces which results in steric hinderance of complement fixation when more than one type of antibody is present in an antiserum. Both the unusual antiserum and unusual platelet may provide further information concerning mechanisms of complement fixation. (John Gockerman and N. Raphael Shulman).

6. Histochemical studies of megakaryocytes and platelets

It has generally been suspected that intracellular concentrations of metabolically active substances and of enzymes involved in cellular functions may be related to the degree of maturity of the megakaryocyte and to the age of platelets. There is very little information in the literature concerning qualitative or quanitative aspects of measuring intracellular substances in these cells. Utilizing cytochemical techniques that have been applied to leukocytes, acid phosphatase, glycogen, and cytochrom oxidase have been demonstrated cytochemically in megakaryocytes and platelets. In the case of acid phosphatase there appears to be an isoenzyme specific for these cells. The staining obtained with each technique has shown a high degree of resolution that permits enzymes to be estimated by a semiquanitative scoring procedure. Current investigations suggest that it will be possible to establish patterns of enzyme distribution in megakaryocytes and platelets that potentially can be correlated with function and age of these cells. (Olivera Markovic and N. Raphael Shulman)

Honors and Awards: None

Publication:

1. Maddrey, W., Saito, S., Shulman, N. R., and Klatskin, G.: Significance of Australia Antigen in Primary Biliary Cirrhosis. Ann. Int. Med. 76:705, 1972.

ASSOCIATE DIRECTOR'S REPORT

Reorganization of NIAMD Extramural Programs

At last year's appropriations hearings it was agreed that an Assistant or Associate Director for Digestive Diseases and Nutrition would be appointed. Dr. Whedon and other NIAMD staff have sought a reorganization of the Institute which would provide greater program identity and innovation in reaching goals important to the areas of Digestive Diseases and Nutrition. After several months of deliberation, the specifics of the plan were brought to the Council for discussion, and were accepted.

The details of the reorganization of the Institute focused about the appointment of an Assistant Director for Digestive Diseases and Nutrition. The present extramural Gastroenterology and Nutrition programs were combined under a new Assistant Director for DD-N, who would provide independent and autonomous administration of these research, training, and fellowship grant programs. An appropriate fraction of NIAMD research, training, and fellowship budgets were to be isolated for use by the DD-N programs independent of the funding of the remaining nine program areas. The Assistant Director for DD-N would be substantively independent and would report directly to the Director, NIAMD. He would collaborate with the Associate Director for Extramural Programs for operational support of DD-N grants. In addition, the Assistant Director for DD-N would develop a contract mechanism to complement grant activities, coordinating these efforts with the existing NIAMD contract operation. The Assistant Director for DD-N also would have responsibility for establishing a clearing house of information and a coordination effort for all of NIH regarding digestive diseases and nutrition.

It was decided that the Associate Director, NIAMD-EP, would temporarily assume the additional function of "Acting Assistant Director for Digestive Diseases and Nutrition." Because of personnel ceiling constraints no additional personnel were able to be assigned to the extramural DD-N effort. Nonetheless, for the overall Institute, administratively and substantively, some progress was made. Recent development of the two new instruments for support in DD-N was completed, and the first round of applications reviewed and funded. A new and augmented Intramural DD-N effort is in operation at Phoenix, Arizona, under Dr. Scott Grundy, chiefly in gall bladder disease. The move to establish a liver unit within the Intramural Clinical Center activities is progressing; laboratories are being constructed. As will be described later, special emphasis has been given to DD-N in funding of research grants. For the first time, contract funds have been used in the DD program to support a controlled cooperative clinical trial treatment of Crohn's Disease.

During the course of the year there was continued progress and thinking concerning reorganization within the entire extramural portion of the Institute. Plans were made for a regrouping of program areas into four major subdivisions or clusters, each with its own Associate Director. It is proposed that the elements of NIAMD which support all Extramural research and training and development activities be reorganized in a manner to allow more effective fulfillment

of its categorical responsibilities. The essence of the proposal is to group the present eleven Extramural program areas into four "clusters" of closely related efforts, and to provide each new program grouping with considerable autonomy and greater freedom to respond to its particular and total program needs. Implicit in the proposal is an opportunity to go beyond the present effective level of selection and management of research grants, fellowships and training grants; inherent is an opportunity for enhanced capability for program analysis, deeper relationships with lay and professional organizations, augmented public informational activities, support of innovative efforts, a more deliberate study of the relationship of grant support to the patient care and educational needs in NIAMD categorical areas, and assumption of greater Institute initiatives. A significant aspect of the proposed reorganization is that each new program grouping would coordinate both grant and contract support of research and training. The proposed reorganization does not alter the Intramural Laboratory and Clinical Research programs which will remain under the leadership of the Director of Intramural Research.

It is proposed that the present eleven program areas be reorganized into four major "Program Areas" as follows:

1. Arthritis, Bone and Skin Diseases
2. Diabetes, Endocrine and Metabolic Diseases
3. Digestive Diseases and Nutrition
4. Kidney and Urologic Diseases (joined with the Artificial Kidney/Cronic Uremia Contract Program)

The location of Blood Diseases (Hematology) has not been decided but could be assigned either to area 1, 2, or 3. The Associate Directors of these programs would have close access to the Director through a Deputy Director for Extramural Programs (Grants and Contracts) of the Director's Office staff.

The reorganization of the Extramural Programs operation into four clusters has major implications concerning allocation of funds to each area. This would bring about completely new situations in budget planning and funding. The problems, although not insurmountable, will require detailed study and resolution.

The very recent passage of H.R. 13591 changes the name of the Institute to "The National Institute of Arthritis, Metabolism, and Digestive Diseases." This bill also establishes an "Associate Director for Digestive Diseases" and a committee of Council composed of members of the Advisory Council who are "outstanding in the diagnosis, prevention and treatment of digestive diseases." Plans for implementation of the provisions of this bill are now being formulated.

Change in NIAMD Principle for Funding Research Grant Applications

In Congressional testimony for the FY 1972 Dr. Whedon and others of the Institute and of the Office of the Director, NIH, have proposed increased program emphasis in the specific areas of digestive diseases and nutrition, renal disease, diabetes, and arthritis. Increased funding was obtained for FY 1972, most of which was with Congressional earmarking (the only earmark resulted

in increased support for renal disease research grants by $520,000). To be consistent, the Institute considered methods for distributing the newly acquired funds in ways to demonstrate that increased emphasis would be given to those areas for which the additional funds had been requested. This is in contrast to past Institute practice of funding a similar percent of each program area's approved applications.

In the discussion, two modifications were recommended by Council. The first was a general one which dealt with "purifying" the group of applications that should be considered in calculating the percentages to be funded; in essence, it was decided that the percentage of approvals funded within a program would be estimated after removing from the calculations those dual applications for which AM was not the primary assignee. Past funding history shows that an extremely small percentage of such grant applications in fact become funded by NIAMD. The Council subscribed to the view that the most equitable handling of program areas would result if only the applications which truly competed for NIAMD funds were considered in estimating the number to be funded. This change in policy will have a significant effect because of the varying numbers of secondary dual assignments to NIAMD in its various program areas and because of the large number of secondary AM assignments. The decision to exclude duals (as well as other reasons for nonpayment) in these calculations was facilitated by a review of Fiscal Year 1969 funds which demonstrated that there was indeed considerable variation between our eleven program areas of the percentages of the approved applications which were funded with AM monies.

The second modification resulted after consideration of several alternative emphasis plans presented by staff. The Council finally recommended a plan that would provide a major emphasis to the areas of digestive disease, nutrition, and renal disease, and a lesser degree of program emphasis to diabetes and arthritis as compared with the remaining six program areas. In the plan adopted the numbers of approved applications funded would be increased in the five program areas to be emphasized so that the percent funded would be roughly 5% greater for the arthritis and diabetes programs and 10% greater for digestive disease, nutrition, and renal disease programs than the percent of grant applications funded in the remaining six unemphasized programs. Although such a change may appear to be small, it is indeed significant and if continued for some years may eventually have major impacts on what NIAMD is supporting. The adoption of this alternative deviates from a long-standing Institute policy. It is believed to be more responsive to the needs and to academic community and Congressional opinion, yet does not deviate significantly from the principle of supporting applications that have been judged to be of high quality.

Review of Program Projects and Categorical Clinical Center Programs

The Council was presented with a detailed fiscal summary of P01-P02 support from 1951 until the present and discussed the importance of these programs. Discussion of program projects indicated that the Council believes that these are legitimate, important instruments which should be maintained. Applicants should be warned, however, that such projects are scrutinized so minutely that they may be less likely to be funded than comparable quality project research applications. Nonetheless, once a principal investigator is aware of this,

he should be free to submit a program project application. The Council believes that the level of support for Categorical Clinical Center programs should "seek its own level"; no active programming should be initiated. Monitoring is required to insure concordance of funds required for existing Center programs and funds earmarked for this activity.

Council Review of Training Grant Applications

Over the past two years, by practicing severe economies within individual grants, the Institute has been able to moderate the decline in number of grants funded with its available money. Having now reached the point at which the level of previously established commitments approximates the expected appropriation, staff presented Council with a proposal to roughly double the recent decline in numbers for this year in an attempt to relieve this intolerable situation in years beyond 1973.

When faced with this fiscal overview, the Council accepted with reluctance the view that the numbers of active training grants during the next fiscal year must be reduced. The Council remained strong in its opinion that every attempt possible should be made to maintain as high as possible the number of active training grants. To this end, it recommended deleting all equipment items costing more than $5,000; limiting training grant personnel increases to a maximum of 110% of prior support for Type 2 applications; and only continuing the award of new applications where opportunity and qualifications were clearly apparent. Council concurred in the staff position that present negotiation to achieve an average 10% dollar reduction in awards should be treated as a maximum, the sacrifice of a few awards being preferable to further increasing the negotiation figure. The Council continues to be distressed by the strong qualifications of several approved applications beyond the funding cutoff level.

Training Stipend Levels

Staff and Council have discussed the problem of disparity between trainee stipends of NIH grants (particularly to clinical departments) and the level of support to house staff within the same institutions. Continued discussion by trainers, training committees, and NIAMD staff as well as by ECEA brought the subject back to Council for repeated discussion.

The Council agreed that the discrepancy between trainee stipends and house staff salaries is a significant and important one, and that this disparity is particularly important to NIAMD because more than 90% of its training grants are in clinical departments where trainees are "in competition" with adjacent chronologically similar house staff. It was a unanimous view that increased stipend levels for trainees (and for postdoctoral and special fellows as well) were desirable and necessary. When asked whether it would be in favor of increasing stipends in the near future to a level on the average some 25 to 30% above current levels, recognizing that there would necessarily have to be a reciprocal reduction in the numbers of training grants, in the average number of trainees per training grant, or by some combination of these, the Council voted ten in favor, two against, and one not voting. The Council recognized the hardship that would be created by this reciprocal reduction in numbers of training grants or trainees, and would hope that additional funds might become

available to preclude that reduction. Nonetheless, should additional funds not be made available, it was the sense of the Council that the importance of increasing trainee stipends was greater than the disadvantage of reciprocally reducing numbers of training grants and/or trainees per grant. No action has been taken. We are awaiting an overall NIH position to be taken on this important matter.

Study of Overlap Between Veterans Administration and NIAMD Training Grants

Veterans Administration support of training as it might overlap with programs in NIAMD was studied. The study was directed at approximately 60 NIAMD training grants being presented to Council this year for funding consideration. Overlap of institutions and personnel was so small as to be inconsequential.

Extension of Clinical Investigator Award and Academic Career Development Award to Other Program Areas of NIAMD-EP

One year ago, two new training instruments were established to support the areas of Digestive Diseases and Nutrition. These were the Clinical Investigator Award in DD-N (K08) and the Academic Career Development Award (K07). When initiated in the areas of Digestive Diseases and Nutrition, it was explicitly stated by Council that these same instruments would be of great value in other program areas as well, and should be extended to other areas at a later date. During the latter part of this year, the Council reviewed the advantages and disadvantages of extending the K07 and K08 awards to other program areas. In essence, the Council believes that these are very valuable instruments and would be of great benefit if they were extended to all program areas of NIAMD-EP. The constraint of lack of increased funds, however, prevents serious consideration of such an expansion at this time. Instead, the Council is in favor of extending these instruments to one additional portion of the Institute's programs, recognizing that this could be accomplished only at the expense of the existing training grant (T01) programs. After consideration of various alternatives, including the extension to those programs with the greatest "shortage" of academicians (estimated by staff to be dermatology, orthopedic surgery, and urologic surgery), the Council recommended that a practical compromise would be to extend the two new instruments only to Program 12, Kidney Disease and Urology. It was the combination of great need for these instruments in that field, and the contribution to a field of clinical disease greatly in need that led to this conclusion. Plans are being developed regarding numbers of such awards, costs, proposal of instructions, guidelines, forms, announcements, etc., which the Institute will forward to Building 1 in its request for permission to extend the Clinical Investigator Award and the Academic Career Development Award to the fields of kidney disease and urology.

Council expressed its opinion that such "New Instruments" as those discussed should be extended further as soon as possible. Special reasons for adopting them in specific programs should be developed as was suggested for the Kidney Disease Program.

NIAMD Experimental Training Programs

During the 1963-67 era of rapid growth for its training support programs the Institute responded to expressions of interest in developing a more coherent training experience for clinical investigators by awarding funds to support a small experimental group of training programs judged potentially to offer significant improvements over the usual categorical research training grant.

As a result of the small size of this experimental group of grants, applications for continuation come to Council's attention only sporadically. Review of renewal applications from two of the five programs led to recommendation by the initial review group (the Institute's Research Career Program Committee) that definitive action be deferred to allow more comprehensive review of the entire program. Dr. Myers served as Council liaison at the meeting of the Institute's Research Career Program Committee at which time a thorough review of the program led to the following conclusions:

1. This type of support was viewed more as pump-priming than as long-term support.

2. The need for experiment in training programs in today's world was considered great. These should be in more than one format (as has been true in the past). A figure of approximately 10 percent was suggested as the fraction of the Institute's training funds that might reasonably be utilized in this way.

3. Of several experimental plans considered, the best defined was an "intercategorical" training grant, an award to be considered provided the applicant can demonstrate significant advantage to a single program spanning two or more of the Institute's several areas of responsibility.

4. New types of programs should be made broadly open for competition.

5. Ongoing programs in the present experimental group would have to compete within this framework.

In keeping with the above expressions Council favored a motion that recommended phasing out of the present Experimental Training grants, unless any one of these should compete successfully within the newer concept.

Role of the RCP Committee in relation to Training and Fellowship Instruments

The NIAMD Research Career Program Committee, augmented with members of training grants committees, had a special meeting in February to discuss the experimental training grants as they relate to regular training grants (T01's), and the interplay with RCDA's, fellowships, Clinical Investigator Awards, and Academic Career Development Awards. In view of the changes in the initial review of RCDA applications, the role of that Committee also was explored.

In a wide-ranging discussion of the role of the various training and fellowship instruments, the assembled group discerned a need for an overview mechanism that would allow the interdigitation of decisions being made or recommended

by initial review groups on all of the training-fellowship instruments. The RCP Committee plus one representative from each of the categorical training committees and one representative from Council agreed that this augmented committee could have an important role in reviewing all recommendations of initial review groups regarding experimental training grants, T01's, F2, F3, K4, K7, and K8 applications. Indeed, it could function as a "sub-Council" which would allow a much more thorough and detailed exploration of the implications of decisions regarding individual applications and assessments of impacts upon the various program areas of NIAMD in a way that cannot be accomplished by the present Council. The recommendations of this "sub-Council," with detailed documentation of their decisions, would then be presented to the Council for it to exert its usual advisory role. Such a system would both augment and diminish the function of Council: regarding training grants, the Council would have much less detailed discussion in reaching decisions than at present. Similarly, it would spend much less time in reviewing the details of individual applications for the Clinical Investigator Award and the Academic Career Development Award. On the other hand, it would gain a review role for postdoctoral and special fellowships and Research Career Development Awards which prior to the present time have not been presented to Council (except as information). It was the sense of NIAMD staff, the Institute Director, and the Council that the review of all training and fellowship instruments by one intermediate body such as this expanded RCP Committee would have a very beneficial effect on the decisions reached. It would allow considerably more rationalization of the decisions made regarding the various training instruments.

Dr. Myers reported on the evolving role of this committee. With study sections now handling the scientific merit review of Research Career Development applications he expressed his opinion that the committee may now expand its deliberative functions as an aid in developing a more coherent perspective on the Institute's educational support activities as described in more detail above. Council expressed its view that the RCP Committee should be encouraged to adopt a "sub-Council" function. Further exploration is in progress to identify means for implementation.

Summary

The Extramural Program continues on its way through a transitional period which involves reorganization, changes in funding patterns, increased emphasis of some programs, and a move into greater analysis of its research and training efforts as they relate to research, training, and patient care needs in its categorical areas of responsibility. Doing so at a time of restricted personnel has made the transition more difficult. New efforts are being pursued with existing personnel. The continued fulfillment of multiple full-time position responsibilities by Dr. Offutt and Mr. Neff reflects the need for increased personnel. The interplay of Dr. Batchelor (the Training Grants Officer), Dr. Michalski (the Research Grants Officer), and their supporting staffs with the Scientific Programs Directors has facilitated the operation of this very large and complex Extramural Program. Dr. Wilford Nusser has continued to carry the difficult role of Acting Scientific Programs Branch Chief during this transitional period. Dr. Offutt's superb overview and management of the grants program as it relates to all the professionals and

to Council remains exemplary. The proposed changes in Extramural Programs described above are looked upon as a potential opportunity for a broad advancement of the Extramural Programs in fulfilling its wide range of categorical interests and responsibilities.

Lionel M. Bernstein, M.D.
Associate Director for Extramural Programs

Researchers in the arthritides have gradually evolved into two major and separate areas, those seeking a microbial organism as the possible cause of arthritis, and those studying the possible role of autoimmune processes in arthritis.

Microbial studies. Preliminary data reported last year indicated possible exclusion of specific viruses from tissue cultures of rheumatoid synovial cells. Such exclusion phenomena are interpreted to indicate the presence of virus or virus-like particles within these cells. Attempts to reproduce these results in other laboratories have been unsuccessful. One group of researchers has reported the presence of a mycoplasma in tissue cultures obtained from the original reporting laboratory, but could demonstrate no viral exclusion. Another group reported the presence of mycoplasma and some apparent viruses in their sample. Identification of the species of mycoplasma has not been completed. The discrepancies in the early reports from these laboratories have stimulated renewed interest in the possible role of microorganisms as the causative or initiating agent in rheumatoid arthritis. Research is continuing to further elucidate a possible relationship between mycoplasma, virus, and arthritis. Attempts to identify a non-human DNA in human arthritis synovial fluid have so far been negative, but refined and more sensitive detection techniques are being adapted to this avenue of research in an effort to detect minute quantities of non-human DNA.

Immunology studies. Research of immune mechanisms is directed toward a better understanding of the immune reaction in non-arthritic individuals as well as the possible role of the various components of the immune system in the arthritides. Studies of the complement system in humans with rheumatoid arthritis have shown, for example, that increased C_9 levels correspond directly with the erythrocyte sedimentation rate regardless of whether the patient is seronegative or seropositive. In contrast, the C_1, C_2, C_3, and C_4 serum levels do not correlate with sedimentation rates. Radiolabelled studies of complement protein C_3 indicate that splitting of this component yields anaphylatoxin C_{3a} and C_{3b}. C_{3b} appears to enhance phagocytosis and release of lysosomal enzymes which may be an important factor in synovial cell damage in rheumatoid arthritis since lysozymes are known to be tissue destructive. C_3 may also produce C_{5a} which possesses both anaphylatoxic and chemotactic activities for human neutrophils.

The role of the immunoglobulins in the immune response, especially as it relates to rheumatoid arthritis or the inflammatory response, is being studied utilizing biochemical and immunological techniques. Cleavage of the molecules through the use of various enzymes and other agents has permitted study of specific fragments, and subsequent characterizations of some of these fragments. Such studies, combined with antigen-antibody studies, electron microscope observations, and amino acid sequencing, have permitted partial reconstruction of the molecular configuration of specific immunoglobulins, as for example the gamma immunoglobulin Eu. Hopefully, continuing research in this area will elucidate the structure and specific binding sites for each of the identifiable immunoglobulins associated with arthritis or the tissue inflammatory response.

Clinical trials. The Cooperating Clinic program is continuing. Studies planned for the current year include (1) a second study of cyclophosphamide in RA, (2) completion of the gold therapy trials, and (3) analysis of the early stage variability study. This was a three-year project covering 104 patients in which approved diagnostic tests were performed on a regimented basis to determine the variability in these measurements, rate of remission, etc., in patients receiving the usual treatment for rheumatoid arthritis. The non-steroidal anti-inflammatory trials have been temporarily postponed in order to concentrate funds and efforts on ongoing trials.

One grantee has recently completed a clinical study on the effectiveness of L-histidine in relieving symptoms in patients with RA. Preliminary results from a controlled double blind study on a restricted number of patients have indicated that patients receiving this drug have shown detectable objective improvements. These results must, however, be evaluated in relationship to somewhat similar results obtained by other investigators on patients receiving only passive-bed-rest treatment. The histidine study is being expanded to include additional patients and possibly to include additional clinics.

Collagen. Major areas of emphasis in collagen research are (1) biosynthesis of collagen, (2) identification of enzymes involved in this process, (3) isolation and identification of protocollagen proline hydroxylases, (4) amino acid sequencing for characterization of the molecular structure of collagen, and (5) analysis of collagen in specific collagen diseases and efforts to identify defects associated with these diseases.

Techniques similar to those used in studies of the immunoglobulins are employed. Recent reports indicate that enzyme defects have been detected in some of the collagen diseases. Data available are still preliminary and further studies are necessary.

Polymyositis. A grantee has demonstrated that muscles from patients with polymyositis when incubated with the patients' own serum lymphocytes, produce cytoxins which result in muscle destruction.

Earmarked funds. Only four of the grants originally funded in 1966 as part of the special earmark funds are still being funded by this Institute. Each of these applications has undergone recent Study Section and Council review and continued funding was recommended.

Wilford L. Nusser, Ph.D.
Arthritis Program Director

Research in Psoriasis

Support of psoriasis-related research has become one of the major priorities of the Dermatology Program area during the past fiscal year. Although the etiology of psoriasis is unknown, current information points to excessive epidermal mitosis as being the final tissue event that accounts for the major clinical expression of this disease, i.e., the formation of scales. Available evidence suggests that interference with epidermal cell replication can satisfactorily control the cutaneous lesions. Identification of other possible means for intervening in the psoriasis process was the major recommendation of the psoriasis research planning meeting held at the Skin and Cancer Hospital of Temple University in November 1970. This preliminary meeting organized by the NIAMD staff was sponsored by the National Psoriasis Foundation, a new voluntary health agency that has worked closely with the NIAMD. Recognizing the serious disabilities resulting from psoriasis and the need for increased research efforts in this disease area, NIAMD sponsored a workshop on "Cell Controls in Psoriasis" held in October, 1971, at the NIH. Scientists of diverse backgrounds but with a common interest in cell controls reviewed and evaluated the possible defects in controls of cell behavior that might be implicated in psoriasis and identified areas of research that appear most relevant to further progress in understanding and controlling the disease. There was general agreement that the workshop provided an opportunity for synthesis and distillation of old and new ideas about psoriasis. In addition to the suggestion that more research be directed to the study of growth regulation in normal and psoriatic skin, participants stressed the importance of utilizing chemotherapeutic agents such as methotrexate that could be applied topically to the psoriatic lesion. Experience gained in the development of drugs for chemotherapy of cancer was considered to be particularly valuable in future development of drugs for psoriasis. That experience facilitates a search for drugs for use in psoriasis. Available are a number of drugs with known cytotoxic action; while several have been found to benefit psoriasis, a number of disadvantages have limited their use. The identification of more effective and more safe drugs is needed. New topically effective drugs might better avoid systemic toxicity. The workshop achieved its objective of focusing present knowledge in the general area of cell controls to the field of psoriasis.

Implementing the recommendations of the participants in the October 1971, psoriasis workshop, NIAMD with the cooperation of the NCI sponsored a Psoriasis Topical Chemotherapy Planning Meeting held in the National Cancer Institute on April 18 and 19, 1972. The specific objective of this meeting was to design and establish a chemotherapy screening program for the topical treatment of psoriasis. Following short presentations and round table discussions, the dermatologists, oncologists and pharmacologists resolved many theoretical and practical aspects of the proposed project. Discussion centered about the use of various known chemotherapeutic agents, the vehicles to be used, the controls, testing procedures, finances, and the personnel participation required. A recommendation will be submitted by this ad hoc advisory group jointly to the NCI and NIAMD to develop a cooperative screening program of chemotherapeutic drugs for the topical therapy of psoriasis.

NIAMD support of psoriasis-related research has increased in the past fiscal year. A new project "Cell Division Control in Normal and Psoriatic Skin" will test the hypothesis that epidermal cell proliferation may be regulated through the cyclic AMP system. By correlating decreases in epidermal cyclic AMP with increased epidermal proliferation as in psoriasis and rises in cyclic AMP with depressed epidermal proliferation (under the influence of epinephrine or theophylline), grantees hope to resolve this important question.

Research in Photobiology

Research is being supported by NIAMD to evaluate the efficacy and safety of high doses of beta-carotene for the amelioration of the photosensitivity in erythropoietic protoporphyria and to study the cellular mechanisms by which carotenoid pigments exert their protective action. Of 30 patients with erythropoietic protoporphyria (EPP) treated with beta-carotene, only one failed to respond; three have shown slight improvement; and 25 have at least tripled their ability to tolerate sun exposure. Some have improved markedly; 11 can stay outdoors six hours or longer. Investigators are currently examining beta-carotene as a photoprotective agent for sunburn in normal individuals. Cellular destruction by visible light is a phenomenon not restricted to any given biological species. Indeed any cell exposed to light is in danger unless the cell contains some kind of protective mechanism. It is now established conclusively that carotenoid pigment is capable of preventing photosensitization not only in bacterial cells but also in animal and plant cells. Grantees suggest that in all cells exposed to light there is a normal photosensitizer/photoprotector balance and that in the cells of patients suffering from photosensitive diseases this balance is altered because of the accumulation of large amounts of photosensitizers. Grantees postulate that the systemic administration of carotenoids may result in the absorption of carotenoids by skin cells thus restoring the proper skin sensitizer/photoprotector balance and thereby preventing further photosensitization. At present, there is no completely effective therapy for the prevention of photosensitization in conditions where photosensitizers activated by light above 4,000 angstroms tend to accumulate in cells; from the currently supported research it appears that systemically administered carotenoids may prove to be such a therapy.

Other grantees are attempting to explain and evaluate the effects of therapeutic agents on epidermal macromolecular metabolism. In guinea pigs, grantees have documented a dramatic, rapid effect of local hydroxyurea of inhibition of epidermal DNA synthesis immediately following its penetration into the epidermis. This inhibition of DNA synthesis has been associated with stimulation of protein synthesis, a finding reminiscent of effects of several other inhibitors of macromolecular metabolism in epidermis. Studies are currently being continued to define its specific site of action. These studies may be of great significance with reference to the therapy of hyperplastic epidermal disease (ranging from psoriasis and ichthyosis to cutaneous neoplasia). They suggest that local antimetabolites could be developed and eventually used without the risk of side effects associated with systemic therapy.

Acne Research

Acne is the most common disease seen in dermatologic practice. Ninety percent of females and almost one hundred percent of males experience acne at some point in their lives. The effects of this condition can range from negligible to devastating. The amount of money spent on over-the-counter acne aids alone amounted to $37,900,000 in 1966. One NIAMD-supported project is attempting to define the pathogenic factors in acne vulgaris in order to improve therapy. Therapeutic agents which either decrease the amount of sebum produced, or prevent the hydrolysis of fatty acids in sebum are of aid in the disease. Continued collaborative studies of the effect of the microflora have shown that aerobic organisms do not change the fatty acid content of sebum. However, there is a direct correlation between the C. acnes population and the level of the free fatty acids. Furthermore, the administration of tetracycline reduces the free fatty acid levels in subjects with a high C. acnes population, but does not produce any change in subjects lacking a significant C. acnes population initially. These studies again indicate the probable importance of C. acnes as a contributor to lipolytic activity.

While various sulfonamides have been ineffective in altering the composition of sebum, the combination of sulfisoxazole with the potentiator, trimethoprim, decreases the free fatty acids in sebum. Thus, there is the possibility of using an antibacterial agent that has a different mode of action than the tetracyclines.

Research in Collagen Disease

New findings have been reported on the possible defects in scleroderma. An investigator reports that the most significant changes in sclerodermatous skin take place at the level of the subcutaneous tissue. There appears to be an inflammatory reaction followed by the replacement of the subcutaneous tissue by newly synthesized connective tissue which consists of fine collagen fibers accompanied by an increase in glycosaminoglycans and numerous fibroblasts. The hypothesis currently being entertained is that an impairment in collagen maturation may be operative in scleroderma.

Research of Occupational Significance

Environmental Injury and Repair of Epidermis is the title of an important ongoing project grant. The investigator is seeking rational and practical methods for prevention and treatment of dermatitis caused by exposure to environmental and occupational agents, such as acids, alkalis, detergents, solvents, etc. Analysis of the fine structural changes produced by occupational exposure in the surface layers of the skin, and of the reactive changes in the epidermis during repair of the damage hopefully will give a scientific basis for the construction of preventive applications. Similarly, treatment of irritant dermatitis can be made more rational. In view of the high incidence of contact dermatitis from occupational and other environmental exposure (estimated at 5-10%), the significance of these studies for general health problems is considerable.

Based upon recent activities within the field of clinical and investigative dermatology, it is anticipated that the dermatology program area of NIAMD-EP should play an important role in the following three areas:

I. Psoriasis Organize and/or participate in an ad hoc advisory group for comprehensive long range plans for psoriasis research. This would appear to be a logical extension of the two psoriasis conferences described above that have been sponsored by the NIAMD. A psoriasis research advisory committee to the NIAMD would be most appropriate and timely as it would reflect the new momentum within the field of psoriasis research. The NIAMD should be prepared to set aside funds to support additional high quality psoriasis research.

II. Acne As mentioned above, acne is the most common disease seen in dermatologic practice. The Dermatology Program sees a need for more investigators interested in acne and the establishment of a controlled population in which acne can be studied. It is proposed that the NIAMD staff, in consultation with its grantees and consultants, study the feasibility of establishing acne research clinics. As in the case of psoriasis, the scientific and technical knowledge has reached a point whereby through proper planning and funding greater control can be exerted over the manifestations of this disease.

III. Eczema The Dermatology Program of the NIAMD has been asked by the Food and Drug Administration, the National Institute of Occupational Safety and Health, and the Eczema Task Force of the National Program for Dermatology to coordinate plans for a state-of-the-art conference on research in primary irritant and allergic contact dermatitis, and on atopic eczema. Examination of the mechanisms is necessary, the occupational and economic implications of this condition which is quite prevalent among middle and lower income groups in industry are obvious. It would be appropriate to consider application of recent immunologic research findings to the prevention and treatment of eczema.

Laurence H. Miller, M.D.
Dermatology Program Director

03 - DIABETES PROGRAM AREA

The major aims of the diabetes program are to define the disease in terms of its causes and the many complications which are associated with it, and to find methods of prevention. To prevent or control diabetes, two goals must be achieved. First, better methods must be established for early detection and prediction of the disease. Second, the fundamental genetic and metabolic causes of the disease must be fully understood so that rational methods of prevention and treatment can replace the present empirical and inadequate approaches.

During FY 1972, the number of approved, on-going research projects was reduced by the limited availability of funds from 126 to 110. All of the 82 continuation projects were funded, many of them at less than the recommended amounts. Only 16 of 28 approved renewal applications could be funded. Of 29 approved new proposals only 12 will be funded. The 17 new proposals which cannot be supported include many excellent projects.

A decrease in training activities in diabetes is also in prospect. Of 18 training grants active during the current year, only 13 will be supported starting July 1, 1972. Only one of three approved renewal applications will be funded. An additional decrease in the number of training grants is predicted for the next year. Of 14 research career development awards (RCDA) active in fiscal year 1972, 11 will remain active during fiscal year 1973. Two new RCDA's will be initiated on July 1, 1972. Seventeen fellowships related to diabetes were active at the beginning of the current year, with no appreciable change expected. The program continues to support one research career award. The continued decrease in these training activities is unfortunate, because they are important sources for staffing medical schools.

The 110 research projects constitute the most direct approach to the many problems associated with diabetes; the training activities supplement these research efforts. A wide range of objectives, approaches and interests are reflected in the research projects; these have been classified for planning purposes into four major groups described below.

Etiology, Pathogenesis and Treatment.

Forty-three of the 110 projects are concerned with the causes and diagnosis of diabetes, the complications of the disease, and the efficacy of current or potential treatments. These projects place emphasis on the study of diabetes in human subjects to complement research in experimental animals. Included are studies of the role of inheritance and diet, changes in metabolism and enzyme concentrations in the diabetic state, characterization of the complications which follow initial development of the disease, study of the mechanism by which known diabetogenic agents cause or simulate the disease, and testing of current and potentially new methods of treatment. Only a few of the projects involve detailed studies of the development of diabetic vascular complications, and much more emphasis on this subject is needed.

About one third of these projects are designed to determine the extent to which hypoglycemic agents (insulin and synthetic drugs), or carefully selected

diets, control the complications developed in the diabetic state (including diabetic retinopathy and neuropathy, glomerulosclerosis, microangiopathy), and effects on longevity.

A second major report on 13 of the projects, constituting a collaborative effort known as the University Group Diabetes Program (UGDP), was published during the year. The Group reported that phenformin (DBI), an antidiabetic drug of the biguanide type, is no more effective than diet alone or diet and insulin in prolonging the life of patients with asymptomatic, adult-onset diabetes. Moreover, phenformin-treated patients had a higher death rate from cardiovascular disease than did patients on diet alone or diet and insulin, as had been reported one year earlier for tolbutamide, an oral antidiabetic drug of the sulfonylurea type. The data also revealed that phenformin was no more effective than the other treatments tested in preventing the occurrence of nonfatal vascular complications associated with diabetes. For these reasons, the use of phenformin has been terminated in the UGDP study, as was the use of tolbutamide earlier. These adverse effects raise serious questions concerning the long-term use of tolbutamide and phenformin therapy. The data throw doubt on the belief that strict regulation of blood glucose levels, per se, (heretofore considered an important therapeutic goal) contributes to longevity of patients with adult-onset diabetes.

Seven approved applications for renewed support and eight for new projects, classified in this group, could not be funded.

Secretion and Activity of Insulin and Related Hormones.

Thirty-one of the 110 projects involve studies related to insulin secretion and activity under a variety of circumstances in normal and diabetic patients and in experimental animals. This includes the histology and physiology of the pancreas, insulin biosynthesis in isolated cells and in secretion granules of the islets of Langerhans and the many factors regulating this synthesis, abnormalities in insulin secretion in the diabetic patient or animal, improved techniques for measuring insulin activity, antibodies and inhibitors which may block insulin action, factors which affect the degradation of insulin, and other hormones which may oppose or supplement the action of insulin.

A project in this area has shown that diabetes is characterized not only by an insulin deficiency but also by a relative or absolute excess of glucagon, a hormone which is antagonistic to several actions of insulin. Secretion of glucagon, which occurs in the alpha cells of the pancreas, is normally suppressed by the high levels of blood sugar found in the diabetic patient. However, the investigator has now reported that normal suppression of glucagon secretion by high levels of blood sugar does not occur if glucose metabolism is blocked, or if severe insulin deficiency is produced. Blockade of glucose metabolism in experimental animals was shown to lead to a striking rise in glucagon levels despite the presence of high levels of blood glucose and insulin! These results suggest that the diabetic patient could benefit directly from intensive research on the control of glucose absorption and metabolism in alpha cells.

Five approved applications for renewed support and five for new projects, classified in this group, could not be funded.

Mechanism of Action of Insulin.

Twenty-seven of the 110 projects are primarily concerned with the precise biological functions of insulin and the mechanisms by which these functions are fulfilled. Subjects under study include the relationship of the structure of insulin to its activity, factors which modify insulin action, the role of other hormones in regulating insulin activity, the mechanism by which insulin controls blood sugar levels, and the effects of insulin on the metabolism and transport of carbohydrates, lipids and proteins. These studies are fundamental in nature and commonly involve investigation at the molecular level.

A project initiated in this group will seek new information on the derangements of carbohydrate metabolism in liver which occur in diabetes. It is hoped that such information will in time provide a basis for new therapies for the disease. The studies will be carried out using suspensions of morphologically intact isolated liver cells which can now be obtained in high yield. Rates of glycolysis, gluconeogenesis, and ketogenesis will be measured in parenchymal cells from normal and diabetic animals, and the response of the cells to insulin will be examined. Metabolic rates will be correlated with levels of key enzymes, and evidence for enzyme induction or repression during long-term incubations will be sought. It is considered that the cell suspensions will prove more valuable than other types of in vitro preparations for the study of experimental diabetes.

Four approved applications for new projects, classified in this important group, could not be funded.

Carbohydrate and Lipid Metabolism.

Nine of the 110 projects are best characterized as comparisons of carbohydrate and lipid metabolism in diabetic vs. normal individuals or animals. Subjects under investigation include pathways for catabolism of carbohydrates and lipids, gluconeogenesis, synthesis and interconversions of fatty acids and lipids, storage and mobilization of lipids in adipose tissues, and the control of such processes by hormones, particularly insulin. Such studies are, of course, essential for a full understanding of the changes which occur in the diabetic state.

An interesting development in this area is that evaluation of the effect of a high carbohydrate diet in patients with mild diabetes has revealed that such a regimen lowers fasting blood glucose levels and improves glucose tolerance without altering total insulin secretion. The results of this study should have implications for the optimal diet for patients with mild diabetes who usually are treated with "diet alone," commonly with restriction of carbohydrate intake.

No approved competing applications, classified in this group, were assigned to the program during the current year.

Lemar F. Remmert, Ph.D.
Diabetes Program Director

The Nobel prize for Physiology and Medicine was awarded in 1971 to an endocrinologist, Dr. Earl Sutherland of Vanderbilt University. Dr. Sutherland was awarded the prize for discovering that a compound, cyclic adenosine monophosphate, (cAMP) acts within cells as a so-called "Second-messenger." Various hormones serve as first messengers which appear to act at cell surfaces. This action stimulates the second messenger within the cell to transfer the message to the cell machinery, which reacts in a specific manner, depending on both the type of stimulating hormone and the type of second messenger.

Another second messenger has received much attention recently. This is "Sulfation factor," a substance which seems to serve as the second messenger for growth hormone. It has been partially purified from plasma, and has been shown to have activity related to sulfation of cartilage as the name implies, as well as other growth mediating effects. For this reason, the term "Somatomedin" has been proposed as the new name for the factor by AM grantees working in the field.

In investigations into the mode of action of hormones, much of the exciting work is at the sub-cellular level. Studies of hormonal activity at the membrane level involve the extraction and characterization of "Binding sites." Because hormones act specifically on some cells, but not on others, there must be a selective process which accepts one hormone while excluding others, and initiates activity at the cell surface. In the process of binding, the hormone is inactivated and probably is degraded at the binding site (or nearby) by specific enzymes. This theory has been advanced by AM grantee Henry Lauson, specifically for the hormone vasopressin. He believes that inactivation of vasopressin occurs at binding sites in arteries and arterioles throughout the body. Although definitive proof is lacking at this time, the evidence is stimulating further work in the area.

Hypothalamic Pituitary Interactions

In recent years attention has been given to factors produced in the hypothalamic area of the brain, which cause the release of hormones from the pituitary gland. These releasing factors are small polypeptides. The first to be characterized and synthesized was thyrotropin releasing factor (TRF). Studies in man by AM grantee John Wilber now show that TRF also causes release of the pituitary hormone prolactin. A separate hypothalamic factor, PIF, is known to inhibit prolactin release. These findings indicate a hypothalamic check and balance mechanism for release and inhibition of prolactin.

Other studies on TRF action by Dr. Wilber are aimed at determining the binding site of this hormone. He has shown selective TRF binding on the plasma membrane fraction from the pituitary.

Thyroid Gland

Basic studies regarding mechanisms of hormone action ultimately are of value in the diagnosis and treatment of human disease. A case in point is the

studies of Dr. Robert Utiger who was one of the co-workers in some of the TRF described above. He has utilized TRF in clinical studies to show the type of malfunction of the pituitary which leads to hypothyroidism. In addition to clinical studies Dr. Utiger continues basic research. He has developed radioimmunoassay methods for the measurement of several of the thyroid hormones and for TRF. With these very sensitive tools, studies can show the interrelationships of the hypothalamus, pituitary and thyroid.

There are still areas of the world where endemic goiter is a problem. One such area is Colombia, South America. AM grantee Dr. Eduardo Gaitan has been studying this problem for a number of years. In an epidemiological study, Gaitan was able to show that although iodine intake and excretion appeared to be within normal limits, there was still a high incidence of goiter in the population in certain areas of Colombia. He has been able to identify the goiterogen as a substance in the drinking water, and is trying to determine its chemical structure.

In controlled studies using rats who drink water with and without the suspected material, he has confirmed that the water borne material is indeed responsible for the disease.

Parathyroid

A complication of hemodialysis in patients with chronic renal failure, is hypersecretion of the parathyroid gland with attendant loss of bone calcium and pain. Studies reported by Goldsmith and Arnaud show that this effect can be reversed by increasing the amount of calcium in dialyzing fluid and decreasing the amount of phosphate ion in the patient's plasma. The role of parathormone was shown by analysis of patients' blood levels of parathormone using a very sensitive radioimmunoassay technique. Following the treatment described, the patients reported less bone pain and the blood chemistry was more nearly normal.

Adrenal Glands

The hormones of the adrenal cortex and of the gonads are steroids. They are produced in the glands by a complex series of chemical reactions which takes them from cholesterol to their final end product. The production of adrenal steroids is controlled in part by the pituitary hormone, ACTH. A model of ACTH action has been proposed by grantee Seymour Koritz. He has evidence that during the conversion of cholesterol to pregnenolone, ACTH acts to keep the pregnenolone moving away from the mitochondria where the reaction occurs. In the absence of ACTH pregnenolone stays at the mitochondria, thus "backing-up traffic," and greatly slowing the reaction rate. ACTH appears to act as a traffic policeman in that it keeps the reaction moving and prevents a traffic jam at the mitochondria, and thus stimulates more steroid hormone production.

Another grantee, Dr. T.T. Tchen while studying the effects of ACTH and melanocyte stimulating hormone (MSH) on the differentiation of melanoblasts to melanocytes has shown that the reaction is mediated by cyclic AMP. It is his opinion that this is the first time that cAMP has been shown to be the

"inducer" in vertebrate embryology. This information may prove extremely interesting and valuable to researchers in the fields of embryology and endocrinology.

General Comments

The NIAMD supports a wide range of basic and clinical studies in eleven program areas. It is of interest to note overlap between program areas, as for example the hemodialysis - parathyroid study reported above. Another example involves gouty inflammation, a study supported by the Arthritis program of this Institute. It is known that men tend to have gout with a much greater frequency than do women. The grantee, Dr. Weissman, found among other things, that lysosomes with estradiol containing membranes ("Female Lysosomes") did not rupture easily, whereas lysosomes with testosterone containing membranes ("Male Lysosomes") ruptured, and released products contributing to the development of gouty arthritis, by increasing the inflammatory reaction of tissues.

A final note should be added. Throughout this report mention has been made of the use of radioimmunoassay as a sensitive tool in the study of hormones. Within the past month, one of the developers of this technique died while attending scientific meetings. Dr. Solomon Berson, was not an NIAMD grantee (his work was supported through the V.A.), but his influence in the general field of endocrinology was wide spread.

Robert A. Tolman, Ph.D.
Endocrinology Program Director

The Digestive Diseases program (formerly Gastroenterology) encompasses research of organ systems associated with the gastrointestinal tract, including the salivary glands, esophagus, stomach, pancreas, liver, gallbladder, and small and large intestines. Research activity is concerned with the basic aspects of the physiology and biochemistry of disease entities. Efforts include studies of the cause, prevention, diagnosis and treatment of specific diseases such as esophagitis, peptic ulcer, pancreatitis, inflammatory bowel disease, malabsorption syndrome, gallstones, cirrhosis and hepatic coma.

A. PROGRESS IN RESEARCH BY INSTITUTE GRANTEES

1. Ulcerogenic Disease

a. The Gastric Mucosal Barrier and Ulceration

Recently, studies have been completed concerning the interrelationship of the gastric mucosal barrier and gastric irritation and ulceration. It has been demonstrated that interference with normal gastric mucosal blood flow, whether by intraluminal pressure or hypotension, is associated with disruption of the normal mucosal barrier to H^+, which may initiate ulceration. Ulcerations produced by ischemia are pathologically similar to human stress ulcers and may be prevented by adequate intraluminal buffering. Moreover, during shock, sepsis, or trauma, there is far greater back-diffusion and barrier disruption than in normal controls. Also, aspirin and alcohol have been shown to disrupt the human gastric mucosal barrier. Chelation of calcium by EDTA disrupts the mucosal barrier in the rabbit as does indomethacin in the dog. Steroids by themselves do not alter canine gastric permeability but markedly augment the barrier disruption produced by aspirin and bile salts. Using the conscious dog as a model, further studies will explore the anatomic delineation of the functional gastric mucosal barrier, relating cellular metabolism to ultrastructure of the gastric mucosa under a variety of controlled experimental circumstances of barrier disruption. This model will be used to test experimental conditions or drugs which may "tighten" the barrier, and perhaps contribute to prevention of ulceration.

b. Nicotine-Induced Inhibition of Pancreatic Secretion

Cigarette smokers have a higher incidence of duodenal ulcer disease than nonsmokers. Evidence has been obtained in the dog to suggest that it is not an excess of gastric acid secretion, but a diminution of the alkaline secretion of the pancreas which may be ulcerogenic in cigarette smokers. The amount of nicotine needed to inhibit pancreatic secretion when given intravenously or by topical application to the duodenal mucosa corresponded to amounts absorbed during smoking. Nicotine seems to be a specific inhibitor of secretin-stimulated bicarbonate secretion. When pancreatic secretion was stimulated with cholecystokinin, nicotine did not affect volume, bicarbonate or protein output.

2. Pancreatic Disease

a. Zollinger-Ellison Tumor Registry

A Tumor Registry of patients with gastrin-producing islet-cell tumors of the pancreas has been an ongoing project at the Medical College of Wisconsin (formerly Marquette School of Medicine) since 1962. The primary objective of this registry is to study the natural history and pathophysiology of patients with this syndrome. Reports from this registry have formed the basis for much of the current information in the literature and the preferred methods of treatment for this disease. Patient data are obtained through the literature, teaching center surveys, personal referrals, and through communication with physicians when they request bioassay or immunoassays. Only patients with confirmed islet-cell lesions associated with gastric hypersecretion are included. The patient information is stored in computer facilities. Followups are made on a yearly basis and the computer data are updated. Nine hundred cases of the Zollinger-Ellison syndrome are available for study. Nearly two hundred of these patients have had total gastrectomy and many have been followed for 5-10 years. The Tumor Registry includes a biographical file of literature references and reprints of publications related to ulcerogenic tumors of the pancreas.

3. Liver Disease

a. Toxic Liver Necrosis

Carbon tetrachloride is an extremely important hepatotoxin. Studies of the mechanism whereby carbon tetrachloride (CCl_4) acts to produce hepatotoxicity has revealed that it causes peroxidation of the microsomal lipid membrane. These changes on lipid peroxidation are observed well before other changes in liver biochemistry or histology are seen. In contrast, a closely related chemical causing liver injury, chloroform ($CHCl_3$) does not increase lipid peroxidation, nor does ethanol. Additional studies are now screening new drugs in an *in vitro* system before testing compounds *in vivo* for their capacity to stop the progression of the induced lipid peroxidation. In fatal CCl_4 liver injury the lipid peroxidation persists; in non-fatal injury it is reversible. Achievement of control over the course of lipid peroxidation *in vivo* would represent a real advance for basic research in toxic cellular injury, and it might create new foundations for possible clinical applications. A classification of hepatotoxic agents into sub-categories, those which do and those which do not produce lipid peroxidation may be of some aid in eventual understanding of the mechanism of toxicity.

b. Treatment of Acute Hepatic Coma

The treatment of acute hepatic coma is infrequently successful, and there is little agreement as to the best treatment -- exchange transfusions, steroids, hyperimmune globulin, lactulose, etc. Compilation of figures by an NIAMD grantee indicates a mortality of from 60-97%.

Based on recent studies, a new mode of therapy is about to be tested in a prospective double-blind clinical trial. Therapy with L-dopa was tested on 11

patients with unresponsive hepatic coma. Three patients with severe alcoholic hepatitis did not respond, but six out of the remaining eight patients who had been comatose for approximately one week despite intensive therapy, awakened quickly after the first dose of L-dopa, and two others did so after 24 hours.

This treatment was based on the hypothesis that the accumulation of "false" neurochemical transmitters may be responsible for the cardiovascular and neurological complications of hepatic failure. Neurotransmitters such as octopamine formed in the gut, were postulated to be poorly degraded in the presence of impaired hepatic function, to accumulate, and to replace the normal adrenergic transmitters at peripheral and central nerve endings. Octapamine was not found in the blood or urine of normal individuals but was detected in patients in precoma and in hepatic coma. Other unidentified amines are also present in blood and urine of patients in hepatic coma.

Data on rats in which hepatic coma was induced by ligation of the hepatic artery after a portal-caval shunt indeed did show the postulated increase in brain and heart octopamine, a potential "false" transmitter amine, and a concomitant marked decrease in the level of the normal amine, norepinephrine. An unexpected finding was the marked elevation of serotonin. Administration of L-dopa restored the level of brain norepinephrine to normal, probably by displacement of the false neurotransmitter, whose brain level decreased. L-dopa also seemed to restore the abnormally high levels of brain serotonin seen in this syndrome back toward normal, probably by interfering with serotonin synthesis.

c. Neonatal Hyperbilirubinemia

Unconjugated hyperbilirubinemia continues to be a major problem in the care of the newborn infant even though significant progress has been made by the introduction of exchange transfusions, and by the proposed preventive treatment of the pregnant mother with phenobarbital and related compounds which induce production of the conjugating enzymes. Since these measures are not uniformly applicable and the safety of drug treatment of the pregnant mother for the newborn infant has not been established, other methods of treatment are still being sought. Current studies have been investigating the mechanism and safety of the use of phototherapy in this disorder. The photolability of bilirubin has been recognized since 1958 when it was first proposed to treat icteric newborn infants with exposure to intense illumination. The effectiveness of this mode of therapy has subsequently been confirmed by many other laboratories, and phototherapy has become almost a routine procedure in many newborn nurseries. Although light reduces the circulating bilirubin level in nearly all instances, the safety of this procedure has not been established because little is known about the chemical, physiological and pharmacological properties of the breakdown products that are produced on photooxidation of bilirubin.

Additional studies are directed toward elucidating the photochemical reactions and identifying and characterizing the intermediate and end products that are formed in the photodegradation of bilirubin in vitro and in vivo. The information to be obtained will permit the potential toxicity (or lack of toxicity) of the breakdown products to be tested; this is essential before phototherapy can be accepted as a safe as well as effective procedure for the treatment of neonatal hyperbilirubinemia.

4. Biliary Tract Disease

a. Dissolution of Cholesterol Gallstones

Although medical scientists have been looking for methods to dissolve gallstones for many years, there have been no indications until this past year that any medical treatment of gallstones might serve as an alternative to the surgery ultimately performed on one-half of the 800,000 new cases of cholelithiasis which develop each year. Results published this past year showed that long-term administration of a normally occuring bile acid, chenodeoxycholic acid, was able to completely dissolve the stones in one of seven female patients in the study group. Subsequent to the publication, two additional members of the group have shown complete stone dissolution and in a fourth patient, the stones were showing a decrease in size. In three of the subjects, gallstone size did not change.

The markedly reduced bile salt pool, characteristic of patients with cholelithiasis, is expanded after chenodeoxycholic administration, although the pattern of individual primary bile salts is altered. Liver function and morphology remained normal, the only undesirable side effect being a moderate dose-related diarrhea.

Based on the encouraging results of this research, plans are being formulated for initiating a cooperative clinical trial. This is discussed under the section on "Collaborative Research and Contracts."

b. Biliary Atresia

It has been reported recently that relatively large amounts of monohydroxy bile acids have been found in the urine of infants with "biliary atresia" or cholestasis. Cholestasis results from a disturbance in hepatic excretory function characterized by a reduction in bile flow and bile salt excretion, generally associated with elevations of the serum levels of bile salts and the alkaline phophatase group of enzymes. The presence of these bile acids may be indicative of a genetic deficiency accounting for the abnormal synthesis and metabolism of bile salts. Sulfated monohydroxy bile acids may have a significant effect on the transport system for bile acids, thus, retarding bile flow and reducing the enteric circulation of the bile salt pool. Since infants with the cholestatic syndrome synthesize and excrete chenodeoxycholate as well as these monohydroxy bile acids, it is postulated that 7α- and 12α-hydroxylations occur by normal enzyme activity, but that the monohydroxy bile salts are derived from a different precursor source. Further studies are contemplated to study the relationship of these unusual bile acids to the reduced capacity of the liver to excrete the normal bile salt load.

B. PROGRESS IN TRAINING

1. Research Training Programs

During the past year, 36 training grants in digestive diseases have been supported for a total of 2.0 million dollars. Ninety-seven trainees have received training in research, teaching and special clinical procedures. For the year starting July 1, 1972, only 31 training grants will be supported. The continuing steady decline in number of active programs occurs at a time when there is an increased demand for faculty and academicians in gastroenterology and hepatology. There is critical need for additional funding for the support of both renewal and new training programs. For the fiscal year 1972, thirteen training grant applications in digestive disease were reviewed by the training grants committee. Of these, ten were applications for the renewal of currently active programs and three were for the initiation of new programs. Although seven of the renewal applications were approved, only two will be awarded. Of the three applications submitted for new training programs, two were approved, but only one will be awarded. Of those six approved applications that will not be funded, four were judged by the review committee to be definitely worthy of support and accorded a ranking score that heretofore was within the range of funding. Continued budgetary restrictions will lead to further restriction of training programs and will have adverse effects on medical education and the national health care service problem.

2. Clinical Investigator Award and the Academic Career Development Award

As described in last year's Annual Report, the NIAMD extramural program initiated two new support instruments for training in the areas of digestive diseases and nutrition; the three-year Clinical Investigator Award (CIA) and the five-year Academic Career Development Award (ACDA). The purpose of the Clinical Investigator Award is to provide the opportunity for promising young medical scientists with demonstrated aptitude in research, under the supervision of appropriate sponsors, to develop fully into independent investigators. This award is expected to provide a stimulus for future faculty in digestive diseases and nutrition within health professional institutions in the country. The aim of the Academic Career Development Award is to provide a well trained young medical scientist with an opportunity to develop the qualifications necessary for an established academic position in digestive diseases or nutrition while concomitantly providing an institution with a demonstrated need, the academic leadership required to initiate or augment essential teaching, research and clinical activities in these areas. An unexpectedly large number of applications were received for the first competition for these awards in spite of the very short period between the June 1 announcement and the September 1 deadline for receipt of applications. A total of 51 applications (20 for the CIA and 31 for the ACDA) were reviewed by the Gastroenterology and Nutrition Training Grants Committee at its October 1971 meeting and by NAAMD Council in November. In the Digestive Diseases program area, six of the 14 applications for the CIA were approved (43%) and five were funded. Six of 17 applications for the ACDA were approved (35%) and five were funded. In the Nutrition program area five of the six applications for the CIA were approved (83%) and four were funded. Two of the 14 applications for the ACDA were approved (14%) of which one was funded.

The Institute has rewritten the instructions for applicants for these two awards, and plans to hold biannual competitions, to be reviewed at the November and June NAAMD Councils. These awards have met with such enthusiasm that the Institute is now planning to propose a similar program in renal diseases.

C. COLLABORATIVE RESEARCH AND CONTRACTS

1. Current Contracts

a. National Cooperative Crohn's Disease Study

Inflammatory bowel disease is a prevalent gastrointestinal disease entity in the United States. NIAMD is the responsible agency of NIH for the support of investigative effort concerned with the etiology, the diagnosis, and therapy of digestive diseases. The incidence of Crohn's disease is increasing in the United States and is now a significant national health problem. Because of its chronicity and the tendency for recurrence following surgery, there is a desperate need for effective medical therapy. Prednisone has been the most frequently used therapy, either alone or in conjunction with salicylazosulfapyridine (Azulfidine). The incidence of steroid side effects is significant. Even less well defined is the therapeutic value of salicylazosulfapyridine. Another drug that is increasingly being used is azathioprine (Imuran), an immunosuppresive agent with varying success rates and equivocal results. There is considerable concern regarding its potential hazards. The length of treatment, dosage, concurrent use of other drugs, toxic and other long-term effects of this drug, and criteria for disease activity indicating or justifying the use of azathioprine have not yet been established, despite which the usage of Imuran is increasing. A controlled study of the value of prednisone, salicylazosulfapyridine and azathioprine is needed to provide the answers to the above questions.

A contract study was initiated in April 1972 to determine the relative therapeutic efficacy of prednisone, azathioprine, and salicylazosulfapyridine in Crohn's disease of the small intestine, colon, or both. A double-blind, randomized, controlled trial will be carried out by cooperating university medical centers. The effects of these drugs will be studied in 1) patients with active and symptomatic disease, and 2) patients whose disease is clinically quiescent. Selection of patients will be based on rigid criteria. The effect of treatment regimens on symptoms, physical findings, laboratory tests, and X-rays will be assessed serially over defined periods for up to two years.

The prime contractor is the University of Colorado, Denver, Colorado. The projected period of performance is four years, with an initial cost of $660,000 for the support of a coordinating center at the University of Colorado Medical Center, and eight cooperating centers. These cooperating centers include: Universities of Colorado, Denver; Missouri, Columbia; Iowa, Iowa City; Ohio State University Research Foundation, Columbus; Vermont, Burlington; Pennsylvania, Philadelphia; Oregon, Portland; Beth Israel Hospital, Boston, Massachusetts.

The study will be augmented by an additional six centers if, after 4-8 months of experience, it is shown that there is need for additional patient accessions.

Statistical services will be provided to the contractor by the Midwest Research Services Center, Hines VA, Hines, Illinois. Such services have been arranged through an interagency agreement between the NIAMD and the VA. It is anticipated that the study will take four years to complete, three years for patient accession and study by the Cooperating Centers, and one additional year for completion of statistical analyses.

2. Future Contracts

a. Gallstone Dissolution by Bile Acid Feeding

Jointly, the intramural and extramural staffs of NIAMD recently have taken the initiative to formulate the requirements for a cooperative controlled clinical trial to test the efficacy and safety of oral bile acid therapy in the dissolution of cholesterol gallstones. An advisory group of scientists knowledgeable in the field of bile acid metabolism, gallstone research, and related areas were convened to consider the need for and the aims, scope, and design of an appropriate controlled study. The recommendations of the advisers were: 1) a well-designed multi-center trial should be carried out in the future not only to evaluate the efficacy of oral bile acids for the dissolution of cholesterol gallstones, but to forestall the premature widespread and indiscriminate use of bile acids for the treatment of gallstones, and 2) the controlled clinical trial should encompass a short-term study of the efficacy and safety of two selected bile acids, cholic acid and chenodeoxycholic acid, to be followed, if the treatment is found effective, by a long-term study to determine incidence of stone recurrence and long-term safety.

In the near future, it is planned to fund a contract study for a double-blind, randomized, controlled trial involving approximately twelve cooperating centers directed by a central committee and advisory board, with appropriate statistical and laboratory support. The first phase of the study will be comprised of the accession of patients, two years on the drug or placebo regimen, and analysis of data. Phase One probably will require a period of three years. During this phase, ancillary studies related to the basic understanding of the disease mechanism, drug action, and potential toxicity will be performed with the test subjects or equivalent patient populations.

D. FUTURE PLANS

In the past few decades, considerable effort and funds have been expended in the support of basic and clinical research related to the understanding of gastrointestinal and liver diseases. Although considerable information has been derived from these programs there are a great number of unanswered questions regarding the basic pathologic mechanisms, the etiology, the diagnosis, and the therapy of numerous highly prevalent disease entities related to the liver and gastrointestinal tract. The major source of support has been provided through the grant-in-aid instruments of the National Institutes of Health. These individual research programs, however excellent, have not met all of the needs of the field. Expansion of scope and effort can be reached by the initiation and support of research contracts. Through the use of continuing review groups consisting of selected renowned experts in their respective research areas, promising and important gap areas where too little effort is

being expended, would be identified. Research contracts initiated and supported by NIAMD would serve to pursue targeted research, fill gaps in our knowledge of digestive diseases, and stimulate the interest of competent investigators to change the direction of their efforts. Appropriate review processes would certify that the research effort would be of high quality. Publications and information forthcoming from such contract efforts would stimulate the generation of research grant applications for the sustained support of multiple long ranged research programs.

Current approaches to major health-related problems could be enhanced to gain significant advances in our knowledge of the etiology of gastrointestinal and liver diseases. Because of the complex nature of many of the digestive diseases, additional progress may be achieved through the initiation and support of interdisciplinary programs of research. Other programs worthy of consideration in the future plans of the digestive diseases program are the support of study centers for selected digestive diseases that are acute and life threatening. These "centers" would execute research projects, evaluate new modalities of therapy and prophylaxis, and serve as training centers.

George Kitzes, Ph.D.
Digestive Diseases Program Director

Introduction

Grants supported by the National Institute of Arthritis and Metabolic Diseases in this fiscal year within the framework of the Hematology Program cover a wide range of projects but may be characterized as either working towards control of blood disease or utilizing blood as a biological tool to study other biomedical problems. This year the support of research in hematology has shown a slight upward trend in total number of projects (about a 10% increase) with added impetus in the study of hemoglobins and blood cell membranes. Applications dealing with hemoglobin S have almost doubled in number over those reviewed in the period June 1970 - June 1971, following the President's emphasis on sickle cell disease in his health message. With the growing realization that many diseases may involve cell surface alterations, the study of cell membranes is a growing area of biomedical research. Since the blood cell possesses the most accessible plasma membrane lending itself to quantitative study, it is not surprising that the number of "membrane projects" supported in hematology is also increasing. To emphasize the unique role of the blood cell in this regard, a section of this report will highlight various kinds of membrane projects underway and point out some recent accomplishments.

Three general directions of the Hematology Program area research efforts will be described through representative projects. The section on Erythropoiesis encompasses work on processes with their modifying nutritional, gastrointestinal and hormonal mechanisms that are responsible for maintaining levels of circulating red cells and hemoglobin optimal for the transport of oxygen. Regulation of white cell production and leukocyte competence in health and disease are included under the section on Leukocytes; Immunity and Transplantation deals with projects studying the molecular basis of the immune response that is important to the understanding of autoimmune aberrations that may cause connective tissue disease, and to the successful utilization of tissue and organ transplantation of the bone marrow, liver and kidney.

Erythropoiesis

Vitamin B_{12} and folic acid - Although nutritional deficiencies of vitamin B_{12} and folate may limit erythropoiesis much less frequently than iron deficiency, they are important because they do impair the viability of maturing cellular elements. Ethanol appears to accelerate the megaloblastic change seen in folic acid deficiency states and studies with alcoholics are yielding significant results. NIAMD investigators have shown that the curve for serum folate depletion in alcoholics is biphasic, and that bone marrow changes occur only after serum folate levels plateau. This establishes the critical role of folate stores in delaying the onset of anemia.

The past year brought the first demonstration of a specific carrier-mediated transport of a macromolecule into the mucosal cells of the intestinal tract. Analysis of ileal cellular debris and mitochrondrial fractions showed a vitamin B_{12}-intrinsic factor complex bound to these subcellular fractions

having entered the cell while attached to some part of the intrinsic factor molecule.

Iron - Studies on the transport and utilization of iron continue with new emphasis on the physical-chemical behavior of iron. Studies of iron binding by lactoferrin and transferrin may increase understanding of the mechanism of iron overloading. The role of iron overload in hemachromatosis must be clarified before fortification of food with iron is implemented.

Hemoglobin

An AM investigator recently demonstrated that adult hemoglobin could be synthesized by reticulocytes obtained from the human fetus at midtrimester. He showed that 9-18 week old fetuses synthesize 8-14% of their total hemoglobin in the adult form. This finding has great implications for the antenatal detection of hemoglobinopathies which requires the genetic expression in the adult form. Obtaining a 10 microliter sample from the fetus in a safe manner remains an operational problem.

Unstable hemoglobins are of pharmacogenetic importance since unoxygenated hemoglobin has increased susceptibility to the oxidant effects of certain drugs. Sulfisoxazole, primaquine and phenacetin may produce potentially harmful levels of noxious metabolites leading to hemolysis in susceptible persons. Although the genetic incidence of hemoglobins characterized by molecular instability, such as Zürich, H, Torino and Köln, is relatively low, a recent study supported by NIAMD has shown Hemoglobin Köln disease occurring as a fresh mutation in which hemolysis was, as expected, accelerated by primaquine.

Work supported on red-cell glycolytic intermediates and oxygen transport has resulted in a likely explanation of developing muscle weakness, anorexia and malaise in phosphate-depletion syndrome. The regulatory role of serum inorganic phosphate concentration in the in vivo glucose metabolism of the red cell has been shown to influence levels of 2,3 -DPG and adenosine triphosphate. Hypophosphatemia leads to reduced levels of these cellular intermediates. This was shown to yield a striking increase of RBC oxygen affinity with the subsequent reduction of oxygen delivery to the tissues. This is thought to account for the symptomology.

Despite the relatively high incidence of hemoglobin Barts in 2-7% in newborn American Negro infants, indicating deficient α-chain synthesis, hemoglobin H disease and hydrops fetalis due to homozygous α-thalassemia have not been reported in this group. Family studies of globin synthesis presented findings differing from those previously described in other racial groups that may explain this. The α/β ratios of globin synthesis in these families indicate a less severe biochemical defect than that determined in Italian and Chinese patients with α-thalassemia syndromes.

In β -thalassemia where there is deficient synthesis of normal adult hemoglobin due to a reduced rate of production of β -globin chains, the process of messenger RNA (m-RNA) translation has been suggested as a possible point of delay. Translation times for the β-polypeptide chain in the

reticulocytes of patients with Cooley's anemia, thalassemia intermedia, sickle thalassemia, sickle cell anemia, hemoglobin C disease and in hemolytic anemias not associated with a hemoglobinopathy however, have not shown a delay. Indeed in several specimens from thalassemics the translation time was even shorter than in nonthalassemics. These results suggest that the defect is due to impaired initiation of chain assembly or a quantitative deficiency in m-RNA and could serve to focus future research in this area.

Leukocytes

Hydrogen Peroxide Production - The polymorphonuclear (PMN) cells from patients with chronic granulomatous disease have been found to lack the ability to produce peroxide. While normal, phagocytosing leukocytes caused oxidant injury in a test system of G-6-PD deficient red cells, PMN cells from affected persons did not. It has been found that normal PMN cells generate hydrogen peroxide during phagocytosis which depletes reduced glutathione in the red cell resulting in its rapid removal from the circulation by the spleen or liver. These findings may provide clues to the loss of function of the PMN cell in chronic granulomatous disease.

Leukocyte Differentiation - Studying granulopoiesin with acute myelogenous leukemic (AML) and pre-leukemic marrows, a team of investigators has found that the leukemic marrow cells are not capable of producing colonies that differentiate to mature neutrophils without the colony stimulating activity (CSA) provided by normal peripheral white cells. Further, they found that cell proliferation causes depletion of CSA in vitro and theorized that leukemic marrow cells consumed excessive amounts of CSA; however, subsequent experiments mixing normal and leukemic cells were unable to prove that leukemic cells could deplete the medium of CSA enough to diminish normal colony-forming capacity. Further work with functional activities of normal and leukemic cells led to findings suggesting that AML marrow has coexisting populations of cells; normal clones which appear during remission and provide CSA, and a leukemic clone which predominates in relapse and which lacks CSA.

In addition, the leukemic cells used in characterizing the functions of normal cells, when they contain the Philadelphia (Ph^1) chromosome, may also be followed in culture utilizing the chromosome as a marker. With this technique, an investigator has gathered additional evidence that monocytes and neutrophils share a common progenitor stem cell.

Hypoplastic Marrow - The effects of herbicides on the ability of the marrow to proliferate have been assessed utilizing an indirect method. DNA synthesis was measured in leukocyte cultures and high rates of inhibition were found with Picloram, Paraquat and 2,4,D. Much investigation regarding the role of environmental factors in marrow hypoplasia remains to be done. New techniques including long-term marrow culture and new concepts are needed to open up this area and establish clear lines of differential diagnosis.

Immunity and Transplantation

Autoimmunity - Investigation of the role of IgA in the development of autoimmune disorders has produced new insight into the etiology of auto-aggressive phenomena. In a clinical study of 30 patients with isolated IgA deficiency more than one-half had some sort of autoimmune phenomena. Reduced serum levels, or both secretory and serum deficiency could be shown, but not secretory deficiency alone. When celiac disease could be associated with selective IgA deficiency, a basement membrane antibody reacting with intestine, bile canalicula and renal tubules could be demonstrated that was not present when IgA levels were normal. These observations show the key role of the IgA gastrointestinal barrier in the prevention of autoimmune phenomena. The demonstration of antibodies to dietary proteins in some of these patients that cross-react with antibodies to self-components suggests that the sources of the autoantibodies may derive from the gastrointestinal tract.

Another study by this same investigator provides the first direct evidence that serum-sickness nephritis can be caused by antibody-antigen complexes. Elution of protein from the kidneys of a patient who acquired bacterial endocarditis secondary to enterococcal infection and died in renal failure revealed anti-enteroccoccal and anti-glomerular basement membrane antibody of the IgG class. The infectious agent acted as an antigen and the development of anti-glomerular antibody was probably a secondary manifestation capable of perpetuating the disease.

Recent work on the mechanism of antibody elicitation points up that control of autoantibody production is exhibited by the tolerance or unresponsiveness of thymus (T) cells to self antigens and by the control these T cells have over their blood (B) cell counterparts.

Complement - Low serum levels of $C\bar{1}$ inhibitor have been discovered in most patients with hereditary angioneurotic edema. Although studies of the liver with fluorescent antibodies showed normal synthesis of complement component C4 and transferrin, in this disorder a biosynthetic error was evident in that decreased $C\bar{1}$ inhibitor was produced.

Transplantation - Finding that there is less deposition of immunoglobulins and complement in liver transplants than in renal and cardiac allografts, mechanisms of cell-mediated immunity are being viewed by investigators with more enthusiasm than those of humoral immunity.

Two new developments hold promise of success in counteracting organ rejection. In a most important development in immunosuppression since the introduction of anti-lymphocyte globin in 1966, an organ transplant team found that the use of cyclophosphamide gives as good or even a better chance of tissue take while eliminating the liver damage seen with the use of azathioprine in kidney recipients. In the other project, a blocking factor has been found in the serum of tetraparental mice that infers immunologic non-reactivity on them. These unique animal models derived from four parents may avoid autoimmunity in this manner and tolerate foreign tissues by virtue of the protective action of this factor.

Further study of this blocking factor may give insight in cancer immunology since it has been shown that although the immune system retains its ability to destroy tumors, blocking factor interferes with this destruction.

Retrospective studies of a large series of serum samples from recipients of kidney transplants are under way with hopes that HLA systems unique to kidney tissue may be found. Improved donor-recipient matching could result from characterizing more specific histocompatibility antigen systems.

A new technique of transplanting thymus tissues into thymus deficient youths has resulted in the stimulation of bone marrow and subsequent control of infections that have often been fatal in these patients. Thymus tissue taken from 12 to 20 week old fetuses and injected into the patients' thigh muscles has permitted the patients' recovery from pneumonia and vaccination infection and although eventually rejected may continue to confer immunity for as long as ten years.

The area of bone marrow transplantation on the other hand shows little promise of widespread clinical application principally because graft-vs-host disease develops almost uniformly in transplant recipients with fatal results. Projects showing potential for solving the immunologic problems involved in marrow transplant survival will be sought and encouraged as a step towards making the technique more widely available to patients with hemoglobinopathies and immune deficiencies.

Blood Cell Membranes

The red cell membrane is studied as the site of the primary lesion in disease (hemolytic anemia) and as a dynamic model of the selectively permeable barrier that protects the internal components of all cells. In hemolytic anemia premature destruction of circulating red cells results from abnormalities associated with the red-cell membrane such as defects in shape, plasticity or permeability, decreased protein sulfhydryl reactivity, altered lipid composition or interaction with immunoglobins or complement. Representative projects studying the membrane as the site of the lesion include studies of the cell's inability to undergo plastic deformation as a cause of hemolysis in hereditary sperocytosis (HS), elliptocytosis, acanthocytosis and with spur cells in patients with liver disease. The effects of steroids and sterols on the red cell's shape, membrane permeability and filterability are under investigation as therapeutic modalities.

Studies demonstrating a genetic error in HS membrane protein which prevents its aggregation into fibrillar or even contractile structures and thereby produces the abnormal shape and rigidity and reduces the survival time of the hereditary spherocyte may have broader relevance in diseases such as elasto-collagen disorders, familial myocardopathy and certain genetic neurological disorders.

By correlating studies of membrane function and lipid metabolism, another investigator is defining the response of the membrane to various hemolytic threats. Lipid renewal systems are characterized by measuring passive

phosphatide exchange and transacylation. Observations on vitamin-E deficient erythrocytes from newborn infants revealed lipid peroxidation when these cells were incubated with hydrogen peroxide. The usual fatty acid composition of the membrane was distorted and this may contribute to membrane permeability changes and eventual hemolysis. Capitalizing on the fact that isoleucine is not incorporated into hemoglobin but is a major constituent of red cell membrane protein, an *in vitro* system for the synthesis of the membrane protein has been set up in which membrane protein from normals and from those with hereditary and acquired abnormalities of the cell membrane will be compared qualitatively and quantitatively.

The physiologic roles of the enzymes phosphoglyceric kinase (PGK) and glyceraldohyde phosphate dehydrogenase (GAPD) that adhere firmly to the interior of the RBC membrane are being investigated with antibodies that inhibit the enzymes. Comparative inhibition rates of reaction mixtures from the cytosol and from the membrane will confirm the location of PGK and GAPD and clarify their role in the membrane's function. When differential binding to membranes is established why some glycolytic enzymes bind substantially to membranes and others do not can be pursued. It is anticipated that identification and localization of membrane-associated glycolytic enzymes could show compartmentalized sources of adenosine triphosphate (ATP) that is needed for membrane events.

Long-term clinical and experimental focus on the cell membranes in auto-immune hemolytic anemia and paroxysmal nocturnal hemoglobinuria (PNH) now under way could yield knowledge pertinent to much broader areas of immunology. The hemolysis of PNH erythrocytes by serum at low ionic strength (the sucrose hemolysis reaction) suggests that a physical alteration of normal serum components may result in antibody-like activity. Through in depth study of these alterations of antibody, reactive sites may be determined.

Ionic transport and cellular metabolism are being studied in red cell models with particular attention to the role of anions in membrane transport. Since anions such as sulfate and phosphate are present in high concentrations in uremic patients, how these anions interact with sodium and potassium may reveal something of their influence in the uremic state. One investigator is using the scanning electron microscope to examine changes in red cell morphology under varying ionic conditions and in the presence of inhibitors of cation transport.

Membrane associated histocompatibility antigens are well defined in leukocytes and platelets and are therefore used in matching donor and recipient in organ transplantation. A new assay has been developed to detect low levels of antibody to histocompatibility antigens that is based on the inhibition of radioactive-labelled antibody binding to lymphocytes. Sensitive and rapid it is 50 fold more sensitive than standard cytotoxicity assays and has demonstrated the presence of antibody in renal transplant failures that was not detectable by cytotoxicity.

A recently developed plasma membrane model that measures the avidity with which cholesterol is bound to serum lipoprotein is the red cell. When

normal red cells are incubated in serum from patients with cholesterol-rich red cells and liver disease they gain free cholesterol from the serum. Sequential absorption with red cells has shown that approximately 1/3 of the free cholesterol in such patients may be loosely bound and thus available for transfer to sites of higher affinity while the remainder is more tightly bound and unavailable.

These investigations of both primary blood cell membrane disease and the membrane as a tool or vehicle for study as a biological system relate to the NIAMD's mission in supporting research in autoimmune disease, renal disease and the metabolic functions of cell systems related to these and other disease processes.

Training

The number of training grants supported in Hematology during fiscal year 1972 remained relatively constant at 36 despite the addition of three programs. Two of the additions represent new programs while the third one was supported again after a year's lapse. One hundred fifteen post-doctoral trainees received support during the fiscal year.

Trainee activities continued in a wide array of projects. There was an increase in the number involving clinical studies in sickle cell anemia. Provision for training in oncology in supported programs varies considerably from institution to institution depending in many cases on local philosophy and personalities. In general, hematologists consider well-rounded training to include that in the area of leukemia with adequate provision for follow-up and evaluation of therapy.

A Career Development awardee was honored for his research in hereditary defects in components of the complement system. The E. Mead Johnson award was made by the American Academy of Pediatrics.

Merilyn C. Hiller
Hematology Program Director

Much of the research supported by the Metabolism Program is of a basic nature concerned with control of the various metabolic pathways of the body. As such, it is also pertinent to other Institute program areas, particularly diabetes, endocrinology, nutrition, and digestive diseases. Hormones are largely responsible for regulating the metabolism of their target organs and some of the most exciting work concerns their mechanisms of action and their relation to the nutritional state of the organism.

Cyclic AMP

As in the past, some of the most important developments continue to come in the field of cyclic nucleotide metabolism. It has been known for some time that cyclic AMP releases protein kinases from inhibition and these in turn activate some enzymes and inactivate others by introducing phosphate groups. Factors controlling these target enzymes thus include the agonist for the adenyl cyclase, the protein kinases, the phosphatases of the target enzymes, the phosphodiesterases hydrolyzing the cyclic nucleotides, the metals required by the various enzymes, and a number of analogs of cyclic AMP which can act as competitive inhibitors to cyclic AMP or as inducers of conformation changes to important enzymes such as the phosphodiesterases. Evidence is mounting that cyclic GMP in some tissues is a second messenger to acetylcholine and that it acts by speeding the hydrolysis of cyclic AMP. Calcium and manganese are being recognized as essential within the cell to the protein kinase reaction and in some cases to the nucleotide cyclase reactions at the cell membranes. The other product of the nucleotide cyclase is inorganic pyrophosphate which is a highly effective permeability factor for calcium or manganese.

Action of Insulin

Like the hormones activating adenyl cyclase, insulin exerts its effect by combining with specific receptors on the cell membrane, resulting in a change in membrane properties. Some of insulin's effects such as that on glucose permeability and protein synthesis can be duplicated with spermine and spermidine. A second major effect is the maintenance of glycogen synthetase in the active unphosphorylated state in the presence of enough cyclic AMP to effect its phosphorylation and thus inactivation. This second messenger is now being revealed as a derivative of cyclic AMP which acts as a highly specific inhibitor.

Insulin and Growth Hormone in Disease

Abnormal response of target tissues to insulin and to growth hormone is seen in many diseases such as diabetes and obesity. Knowledge of the mechanism of action of the hormones should then open up new approaches to management of these diseases by taking into account their second messengers and factors influencing their metabolism. Particular interest has attached to the isolation of the insulin receptors by affinity chromatography, the study of their binding constants, and response to enzymatic digestion. These studies

should be extended to the conditions controlling insulin's production of second messengers as assays for the latter are developed. They should be particularly valuable in understanding abnormal response of target organs to the hormones.

Growth Hormone

The mechanism of action of growth hormone remains obscure but two major effects are recognized. One is the production of sulfation factor by the liver and the second is the sparing of glucose through increased utilization of lipids (growth hormone's anticatabolic effect). Growth hormone release is only partly understood as regards feed-back from its metabolic effects but it does appear to be blocked by the presence in the circulation of sulfation factor. Sulfation factor is incompletely characterized but appears to accompany insulin like activity in a variety of purification procedures and to induce proliferation and production of sulfated acid mucopolysaccharides of connective tissue.

Studies of homocystinuria resulting from a genetic block in the normal cystathionase pathway are providing clues as to the nature of sulfation factor. Under the influence of growth hormone free radicals of ascorbic acid are generated in the liver and catalyse the autoxidation of homocystein to homocysteic acid. The homocysteic acid has many of the properties of sulfation factor including the stimulation of the myointimal cells of the major arteries which can eventually lead to vascular degeneration. Questions are now being asked as to the role of homocysteic acid in the vascular disease of diabetes.

Production of what appears to be sulfation factor is observed in rats infected with an intermediary stage of the cat tapeworm, even in the absence of growth hormone. These animals are characterized by lack of growth hormone release with hypoglycemia and adipose tissue which is refractory to normal lipolytic agents.

Cystic Fibrosis

The pathology of Cystic Fibrosis has been compared both to methionine toxicity and to methionine deficiency which would be possible if the preferential utilization of the amino acid in one pathway led to a deficiency entering others. Methionine has a host of derivatives in the body such as methylated histones, methylated DNA and RNA, methylated contractile proteins and spermine and spermidine (which incorporate the aminopropyl rather than methyl groups). S-adenosyl methionine is the actual transmethylating component but requires enzymes specific for each receptor. The methyl groups are normally restored from the one carbon pool through the folate-vitamin B12 system. Any deficiency in remethylation can result in oxidative or enzymatic destruction of both the adenosyl and methionine moities and resultant deficiency effects.

The fact that Cystic Fibrosis shows many aspects of free radical destruction of membranes suggested that excessive destruction of methionine might be

occurring and inducing a secondary methyl deficiency. This led to a study of the capacity of the fibroblast in tissue culture to incorporate tagged methyl groups of methionine into their messenger RNA. The nucleotides and proteins are synthesized prior to methylation, the latter reaction occurring away from the ribosomes involved in the protein synthesis. Two additional laboratories have now confirmed the original observation that the Cystic Fibrosis fibroblast incorporate only about ten percent as much tagged methyl into transfer RNA as the normal cells while the heterozygotes fall distinctly in between. Short term lymphocyte cultures can also be used for this methyl marker for the gene and is particularly suited to screening for heterozygotes. The fibroblast method makes a sharper distinction between homo and heterozygote and should be suitable for studies by amniocentesis.

Cells from contrast diseases (Hurler's, Hunter's, glycogen storage, etc.) show normal methylation. Less is known about cells incorporating increased amounts of the C14 methyl groups and further study is needed. The highest value yet recorded is 2.45 with many normals around 1. These cells were derived from a man with severe psoriasis. Preliminary results from a genetic survey show a remarkably low incidence of psoriasis amoungst Cystic Fibrosis kindred.

Choline deficiency is a form of methyl deficiency and is characterized by delayed processing of secretory proteins through the endoplasmic reticulum and Golgi apparatus. Thus, a tagged amino acid can be detected in the secreted albumin within minutes of administration while secretion is delayed as much as half an hour in the choline deficient animal. It has now been reported that the Cystic Fibrosis lymphocyte whether separated from the blood or grown in tissue culture contains its beta glucuronidase almost entirely still associated with the microsomes (which include the endoplasmic reticulum and Golgi apparatus) while normal cells have the enzyme predominantly in the lysosomes.

In studies of this type results must be interpreted with particular caution. In general one can rely on tissue culture results only if the culture medium is prepared to identical specifications, cell densities are maintained within in narrow limits and growth conditions are controlled as to temperature, pH, etc. Confidence that an observed effect (such as generation time, protein synthesis, or staining properties) is a direct expression of the gene is increased if the heterozygous cells show similar effects. Metachromasia of the fibroblast in tissue culture has been reliable in some hands but not in others. Depletion of methionine and other methyl products in the culture medium may account for these discrepancies.

These points should be kept in mind in studying cells obtained by biopsy including the lymphocytes from blood. Malabsorption syndromes frequently involve methionine deficiency as well as deficiency of the fat soluble vitamins and these deficiencies can in turn influence the rate of cell differentiation. The methylation of lymphocyte (from the blood rather than tissue culture) RNA may thus be a poor marker for the gene but a good indicator of the condition of the patient. If so it should prove invaluable in evaluation of therapeutic procedures such as nutritional supplementation.

Metabolic Regulation via Methylation

Cyclic nucleotides control metabolism rapidly (fractions of a second) via phosphorylation and dephosphorylation of key enzymes. These reactions require either calcium or manganese as an activating metal so their transport into the cell and subsequent sequestration or release from membranes also participate in the control. Methylation is a much slower process and may occur only in certain phases of the cell cycle and limit cell differentiation until reasonably complete. This would be particularly true of the DNA and histone methylations and probably for the contractile proteins.

The catecholamines are important regulators of cyclic nucleotide metabolism which are in turn regulated (inactivated) by the catechol-o-methyl transferase reaction. The inorganic pyrophosphate product of the adenyl cyclase reaction is a permeability factor for calcium and manganese which are synergistic with the protein kinases. That the availability of methyl groups for the o-methyl transferase reaction is important in regulation is suggested by studies with the pallid mouse which requires markedly increased manganese intake for normal synthesis of connective tissue matrix. This mutant also has a deficiency in amino acid transport into cells and responds to l-DOPA administration like a normal animal which has been depleted of methyl groups by administration of methyl trapping agents such as nicotinamide.

Free Radical Pathology

Much of the cell specific pathology of the body may involve free radical damage with ultimate death to the cell via calcification. Much of the calcium entering the cell to effect the protein kinase reactions is taken up by the mitochondria or endoplasmic reticulum. Any failure of these calcium pumps can result in lethal intracellular levels of calcium. Free radicals are being generated constantly but under controlled conditions so that essential structures are protected. Involved in this control of free radicals are the various electron transport carriers of the endoplasmic reticulum (cytochromes, flavins, metalloproteins, iron-labile sulfur proteins, etc.) as well as such antioxidants as vitamin E, selenium, methionine, etc. The role of selenium in the process is somewhat clearer with the demonstration of its presence in glutathione perioxidase. Selenium is highly effective in attenuating free radicals and selenium has now been identified as a component of glutathione peroxidase. It is possible that its presence does not depend on a codon different from the analagous sulfur amino acid but that the sulfur containing enzyme is destroyed more rapidly.

Such diverse diseases as Cystic Fibrosis, carbon tetrachloride poisoning, Muscular Dystrophy, Wilson's Disease, and iron toxicity may have comparable pathogenesis in the specific cells but be distinguished by the requirement of the cell to process large amounts of specific ions. The parotid secretory granules are built up from the calcium salts while insulin is the zinc salt. As the granule blends with the cell membrane and lyses, the cell must recover the metal to build new granules. The liver is less concerned with packaging its secretory products with a metal but is concerned with storage and release

of iron and copper proteins. Thus, the mechanism of damage to the various cells may have much in common but a defect in handling calcium could cause Cystic Fibrosis, a defect in zinc diabetes, and defects in iron or copper cirrhosis.

James R. Weisiger, Ph.D.
Metabolism Program Director

08 - NUTRITION PROGRAM AREA

The Nutrition program continues to focus on those aspects of basic and clinical nutritional sciences which have particular relevance to improvement of human health and longevity. In the main, these are concerned with advancement of knowledge concerning the role in nutrition of vitamins, trace minerals and elements, proteins and amino acids, fats, fatty acids and carbohydrates, and their relationships to normal and to disordered states of metabolism. In certain of these areas encouraging and important advances have been made during the past fiscal year by investigators whose research has been supported in large part by Institute funds.

Developments and Trends in Supported Research

1. Fat-Soluble Vitamins: In last year's report brief reference was made to an important break-through in vitamin D research demonstrating that the kidney is the site of conversion of vitamin D into the true or metabolically active form of the vitamin, essential for intestinal calcium transport and bone mineral mobilization. Subsequent research has fully confirmed these findings and has demonstrated a role of the kidney in regulation of plasma calcium through a typical "feed-back" mechanism. Chemical characterization of this "true" form of vitamin D is in progress, and its eventual synthesis is anticipated. Its effectiveness in the treatment of renal osteodystrophy, vitamin D-resistant rickets, hypoparathyroidism and related abnormalities of calcium metabolism is under study but availability of this compound proves a limiting factor in such investigations. The metabolic role of other fat-soluble vitamins has also received considerable attention. Recent observations indicate that vitamin K activates a specific protein precursor in the liver, presumably a glycoprotein, which is necessary for the synthesis of prothrombin. Much interest centers around the true nature of the plasma protein involved in the transport of vitamin A, and the role of this vitamin in cellular differentiation and protein synthesis of epithelial and mesenchymal cells. The similarities of action of vitamin E and selenium in protecting cell components against oxidative damage have been somewhat clarified by findings that selenium exerts its effect as an integral part of glutathione peroxidase, whereas vitamin E functions through a different biochemical mechanism.

2. Other Nutritional Factors: Research on water-soluble vitamins, proteins, amino acids, fatty acids and carbohydrates has been quite productive but has resulted in no remarkable advances. However, it is of note that in a 13-year longitudinal study of galactosemic children under dietary therapy there has been observed a remarkable improvement in visual perceptual differentiation and no losses in their IQ.

3. Trace Minerals and Trace Elements in Nutrition: Research in this area has undergone gradual expansion with particular reference to: a) Zinc and its influence on the epidermis, wound healing and reproduction; b) copper as a component of a metalloenzyme involved in collagen formation; c) chromium and its relations to carbohydrate metabolism and diabetes; d) selenium and fatty acid metabolism, and; 3) varied biological interactions between different trace elements, usually involving competition for binding sites on specific proteins. As an example, it has recently been shown that nutritional levels

of selenium can counteract chronic toxicity of methyl mercury in tuna fish, presumably through formation of a complex that reduces the biological effects of each element. Increased knowledge of the biomedical role of trace elements is destined to have an important impact upon the environmental sciences. Regarding trace minerals, major attention has been given to iron absorption and its relation to the high prevalence, world wide, of iron-deficiency anemia in pregnant women and children. Recent observations that meat in the diet greatly enhances the absorption of iron derived from vegetable sources, offers hope for new approaches to this baffling problem.

4. Prevention and Control of Obesity: Overweight and obesity to a degree that interferes with optimal health and longevity, affecting more than 20 per cent of adults in the USA, constitutes a public health problem of major importance and a matter of great concern to the lay public and physicians. In accord with its mission, the NIAMD has supported and continues to support much research directly or indirectly related to this subject. Much attention has been given to morphogenesis of adipose tissue cells, multiple aspects of lipids and lipid metabolism, fasting and periodicity of eating, neural and other factors influencing appetite, endocrine dysfunctions and hereditary factors. Gradual progress has been apparent over past years. However, the payoff has not been adequate. This represents an area where careful planning and organized effort on the part of outstanding investigators in the field are urgently needed. It is pleasing to record that serious consideration is being given to the genesis of a "Special Task Force on Obesity" under the sponsorship of the extramural program of NIAMD.

Support of Research Projects. Compared to FY '70 and FY '71, there has been in FY '73 a small increase in research grant applications (125, 130, and 141, respectively) assigned to the Nutrition area, and a significant increase in the number funded (36, 28, and 47, respectively). Of the grants funded in FY '72, 18 represented new grants and 29 renewals, as compared to 11 and 17 during the previous year. These figures include two supplemental grants, two program project grants and seven grants funded through the US-Japan Cooperative Medical Sciences Program. The total number of active research grants, as of December 1971, was 150.

US-Japan Cooperative Medical Sciences Program

This program funded 18 research grants (13 NIAMD and 5 NICHD) at 90 percent of recommended levels of support. These represented five new grants, six competing renewals and eight non-competing renewals. Because of inadequate funds, one payable new grant and the balance of a partially funded non-competing grant, will have to await funding in FY '73. Since the seven percent reduction in funds allocated to this program in FY '69 there has been a constant inadequacy of funds to support meritorious and highly relevant grant requests, even when other approved grants have been funded at only 80 to 90 percent of recommended levels. As an index of the dire state of program support, it is estimated that if commitments for FY '73 are met in full, no more than one tenth of the total allocation (i.e., about $100,000) will be available for support of research proposals coming to the next three Advisory Council meetings. Support of high quality proposals is especially important during FY '73 in view of the

fact that the Joint Committee of the US-J CMSP is scheduled to review past and current operations of the Malnutrition Panel and decide upon continued support or phase out of its activities. A summary of the research accomplishments supported by this program has been recorded in the "Five Year Report" of the U.S. Malnutrition Panel, published in July 1971.

Training Grants and Fellowships

Two of the 12 training grants in Nutrition were phased out during the year, and an additional one will meet the same fate next year. These grants have supported 24 predoctoral and 22 postdoctoral trainees as compared to 17 and 16, respectively, during FY '71. One new application will be reviewed at June 1972 Council, two new and three renewal applications will come to November 1972 Council. It is hoped that these will prove of high merit and serve to prevent what otherwise will be a serious loss at a time when the need for professionals trained in nutrition is so great. During FY '72 postdoctoral and special fellowships were reduced from eight to six, RCDA's from six to five, and three RCA's have been maintained. The two new training instruments introduced by the Digestive Diseases and Nutrition program area this past year, although well accepted and recognized as having great merit and potential for meeting urgent needs, did not result in as many approved applicants as hoped for in the area of nutrition. Five of six applications for Career Investigator Awards were approved and four were payable; of 14 applications for Academic Career Development Awards, especially designed to support nutrition education, only two were approved and one funded. With much improvement made in the guidelines and more time provided for preparation of applications for the next deadline (June 1, 1972), a much more challenging group of applications is anticipated.

Administrational Developments

The alliance established during FY '72 between the areas of Nutrition and Gastroenterology, constituting the new unit of Digestive Diseases and Nutrition within the extramural program of NIAMD, has proved most rewarding. This has been further enhanced by the appointment of Miss Joan Fredericks as Assistant Program Director in Nutrition. This additional staff strength will greatly augment the collaborative program of DDN and provide the Nutrition Program Director greater opportunities to become more involved with the many challenging aspects of nutrition research and training related to the mission of NIAMD.

Karl E. Mason, Ph.D.
Nutrition Program Director

Development of the electron microscope and subsequent refinements of this instrument have permitted detailed ultrastructural studies of the cell and its constituents. This instrument, along with micropipettes, X-ray diffraction, and crystallography, has extended investigation of the cell to identification of subcellular structures, but has not permitted absolute identification of all of the "inclusion bodies" or other particulate materials of the cell. Recent advances have been made in this area through the use of antigen-antibody and radio-labelling techniques, or via differential ultracentrifugation and isolation of cell constituents in sufficiently large quantities to perform specific biochemical, histochemical, and microanalysis tests for identification.

A grantee has recently adapted the electronprobe so that it can be used as a microanalyzer in conjunction with the conventional or scanning electron microscopes in such a manner that individual electron dense bodies or selected areas (structures) within the cell in ultrathin tissue sections can be analyzed as to elemental content (i.e., Ca++, K+, Na+, etc.).

Preliminary studies have been performed in in situ analysis of mitochondrial granules of hypertrophic chondrocytes. These granules range in size from 500 to 1,000 angstroms. This development has successfully moved elemental analysis from a cellular to a subcellular level. Continued development of this probe and perfecting of techniques for producing ultrathin tissue sections and EM grid preparations which will not interfere with the analysis of particles will permit electronprobe analysis and investigation of intracellular constituents at a level previously impossible.

Tissue contamination which may normally be present in EM studies presented a problem in early pilot studies of this technique. The probe successfully identified silicone and metals as apparent "inclusion bodies" within the cell. Further studies of these unexpected "cellular" elements indicated the diffusion pump and the columns of the EM as the source of these contaminants.

Research and development of the electronprobe and its utilization in intracellular studies are continuing. Production of contaminant-free section demands unusual and elaborate tissue-grid preparation techniques, which have been perfected.

Bone deposition studies. Researchers are in general agreement that hydroxyapatite or similar compounds represent the mature inorganic crystals of bone and tooth enamel. Less agreement is apparent in the theories of actual deposition of this material in tissues.

Research in this area is directed toward the role of the cell in producing materials that will be deposited as hydroxyapatite in specialized areas of the body. Normal tissue fixation of bone-cartilage cells may change the chemical nature of "pre-bone" materials so that basic evaluation of this material becomes exceedingly difficult. Recent advancements in tissue fixation, specifically freezing of the tissue, appear to eliminate the normal fixation artifacts and may permit accurate identification of the sequential steps from the

basic elements to final deposition as hydroxyapatite in tissues. This technique coupled with electron probe microanalysis should help elucidate the sequential evolution of hydroxyapatite in the cell and its subsequent deposition in extra-cellular tissues.

Implants. Recent approval by FDA for use of methylmethacrylate in total hip replacement in humans may have served as an impetus for renewed interest in implant materials and fixation of implants to bones. Unfortunately, progress in this area has been slow over the past few years and no new or innovative procedures or materials have been developed. Research is, however, continuing in development of new materials that more nearly mimic bone or that permit bone and fibroblast tissue ingrowth in the implant to provide a stronger bond between bone and implant. Materials tested to date include, for example, polyvinyl sponges, polyurethanes, ceramics, ceramic plastic composite, various other plastics, and metals. None of these has proved sufficiently attractive to alter present bone implant techniques. Attempts to produce hip and knee joint prostheses using new materials have not been successful to date.

Bone growth - electrical stimulation. Increased rate of bone healing under electrical stimulus has been shown in experimental animals and in at least one human. These results have renewed interest in this area and additional researchers, including engineers, are "looking into" this phenomenon.

Cartilage. Molecule structure of cartilage and collagen is gradually being delineated. Extraction of certain components of these structures via dialysis, chromatography, affinity column chromatography, etc., has permitted use of immunological, histochemical, biochemical, and microscopic techniques in identification of subfractions and smaller components of these tissues.

Models. Researchers are continuing their efforts to identify reliable reproducible models for various skeletal diseases or skeletal defects of humans. Efforts are being made in areas of reproducible fractures of long bone, osteoporosis, scoliosis, and osteoarthritis. Models of scoliosis can be produced surgically. Unfortunately the massive tissue destruction associated with development of this model raises questions as to its relationship to the clinical abnormality. Similar problems are apparent in producing other non-physiological-anatomical models of skeletal disorders.

Bone resorption. 1,25-dehydroxycholecalciferol, which is the most active of the vitamin D metabolites regarding intestinal transport, has recently been shown to be approximately 100 times more potent than 25-hydroxycholecalciferol in its effects upon bone. Other agents under investigation which inhibit bone resorption are antibiotics (methramycin), camptothecin, the vinca alkaloids, and colchicine. Methods by which these materials inhibit bone resorption are uncertain. It has, however, been shown that colchicine inhibits parathyroid-stimulated bone resorption.

Osteoporosis. Research on various drugs in treatment of osteoporosis includes use of fluorides, diphosphonates, phosphates, and oxymetholone. Preliminary results indicate an increased bone density in patients receiving these materials;

however, insufficient data are available on long-term treatment with the agents to determine if the increased density is physiologically sound bone with a concurrent increase in resistance to fracture.

Wilford L. Nusser, Ph.D.
Orthopedics Program Director

A review of research projects supported in this program area during the past year reveals a very impressive application of advances in physics to challenging biological problems. One such technique, intensity fluctuation spectroscopy, goes a considerable step beyond classical X-ray diffraction pattern studies and may be used to study the structural characteristics of actin and myosin. These results allow interpretation of X-ray diffraction patterns obtained from intact muscle at rest and during contraction.

X-ray studies, of course, permit the determination of fixed structural characteristics. Intensity fluctuation spectra of macromolecular systems reflect the dynamic microscopic behavior of the molecular components of such systems and hence allow one to detect changes in the dynamic state arising from configurational or chemical interactions of these molecules. It is the sensitivity of intensity fluctuation spectra to the dynamic state of matter that make it well-suited for studying the contractile events that occur in muscle.

One can only conjecture about the health related significance of these studies. If we understood the molecular basis of muscular contraction and the ultrastructure of the myofibril, it might be possible to synthesize a muscle that would derive its power from normal biological energy sources such as glucose. Such a synthetic muscle would be capable of being supplied by the body's circulation and not provoke an immune response. The use of these synthetic muscles in the fabrication of a prosthetic limb, or a prosthetic heart, or a heart booster pump would be of great significance in the practice of medicine. Admittedly this prospect is far away, but the in vitro synthesis of myosin using polyribosomal particles has already been achieved. Another possible benefit to health-related problems is the introduction of a new analytical technique that requires minute quantities of material and can be used to examine living cells without destroying them. It is entirely possible that I.F. spectroscopy can be useful in the diagnosis of molecular and cellular abnormalities.

The past year has seen the culmination of twelve years' effort in the planning, development, fabrication, installation and calibration of a new physicochemical instrument, the optical diffusiometer. Measurements of ionic movements are now possible of hitherto unapproachable accuracy. The number of scientists whose research depends upon an understanding of the diffusion process is legion, but only a handful of people is engaged in the basic research which develops this understanding. A better understanding of the fundamentals of diffusion in systems composed of several components is important for the solution of a number of biological problems. It will contribute to more accurate determinations of the molecular weights of proteins by the procedure of combing data for sedimentation and diffusion. The coupling of solute flows which occurs in diffusion of systems of three or more components is involved in the transport of substances in living systems and in the non-equilibrium distribution of substances across membranes; at the present time it is not known whether this coupling of flows is a major factor in these phenomena, but it is certain that a complete description of these processes cannot be formulated until more information about this coupling of

flows is obtained. When diffusion occurs in multicomponent systems containing electrolytes, electric potential differences are produced within the system; more knowledge of the fundamental laws of diffusion should contribute to a better insight into ion distributions inside and outside of a living cell, kidney function (both natural and artificial) and nerve potential production.

The study of protein structure changes that occur in various functioning systems, and the ways in which the changes relate to function/change in function has recently acquired a major tool, the differential hydrogen exchange method. This technique allows structure-sensitive measurements on proteins in turbid systems, therefore provides special advantages for the present experiments on the insoluble protein rhodopsin (to be studied still embedded in its normal disk membrane), on other membrane systems, and even on hemoglobin which may be studied in whole red cells (thus avoiding problems such as subunit dissociation at lower protein concentration and the tendency to form methemoglobin during oxy/deoxy transitions both of which have compromised other investigations). Structure change in the photosensitive protein rhodopsin appears to participate in the mechanism of transducing light input into a visual nerve impulse, a kind of mechanism which may (or may not) be general for sensory phenomena. Nucleic acids and polynucleotide models participate in localized, transitory "breathing" derangements of native structure, as do all structured macromolecules, which appears to be important in determining their reactivity in various kinds of interaction, including hydrogen exchange. (The kind of changes termed "breathing", unlike the changes indicated just above, occur naturally, all the time, in the absence of extrinsic effectors.) Cell membranes may utilize structure change in functioning and, in any case, provide a host of interesting and important structure-function problems.

Hydrogen exchange measurements can give special insights into structure change. They allow individual detection of different changes that may occur simultaneously. Hydrogen exchange "characterization" of a structure change can tell the size of the segment experiencing change in terms of number of residues (peptide groups) involved, can identify the kind of change occurring (structured to unstructured, the reverse, change in "breathing"), can measure the structural free energy involved in the change, and can give information related to the amino acid sequence of the segment changed, thus making possible the identification of the changing segment by reference to X-ray models. These advantages suggest that HX studies may contribute significant new information in this area.

Joseph V. Michalski, Ph.D.
Physical Biology and Related Areas Program Director

The extramural program of the National Institute of Arthritis and Metabolic Diseases, through its recently renamed Kidney Disease and Urology Program continues to provide support of projects which relate to basic research and clinical problems of the kidney and the genitourinary tract. Of the total program, the greatest share of support continues to be for physiological and pathophysiological studies of the kidney, while only modest gains in support continue to occur in the urological aspect of the program. The total estimated direct cost support for fiscal year 1972, to be awarded through the Program is anticipated to reach $10.2 million, which is a $2.0 million increase over the actual $8.2 million awarded during fiscal year 1971. The increase can in part be attributed to the $1.5 million which Congress specifically appropriated for this Institute's kidney program during fiscal year 1972.

Kidney Transplantation

Efforts to improve immunosuppression and organ preservation continue to be the primary pursuits of the renal transplant field. A particular emphasis is being made by one transplant group to determine the degree of immunoincompetence induced by commonly utilized immunosuppressive drugs and to correlate this with the appearance of infections and episodes of rejection. To this end a number of investigations are underway on patients to detect their immunocompetence with respect to indifferent antigens and donor antigens, and to screen such patients for viral disease or latent virus.

Kidney transplantation was once felt to be an unsuitable form of treatment for children because they are technically difficult to hemodialyze, there was a fear that they would not grow or mature sexually, and that they would not be able to withstand the psychological stress of dialysis and transplantation. Of sixteen transplants performed on children since August of 1970 by a University of Minnesota group headed by Dr. John S. Najarian, the kidneys of twelve children are functioning normally. A few of the children demonstrated remarkable growth spurts but in most, the growth rate is slightly less than normal children of comparable age. Girls in particular remain short and may become disturbingly Cushingoid.

For many years organ preservation has utilized three main methods: perfusion and hypothermia; perfusion, hypothermia and hyperbaria; and freezing. Some groups have been able to successfully transplant kidneys that were stored for periods exceeding fifty hours. Present goals are to extend kidney preservation to periods of up to two weeks in order to establish a true organ bank. Therefore, the primary thrust in several laboratories is to identify limiting factors which prevent the achievement of longer periods of storage with less functional impairment. Preliminary studies have shown that the isolated perfused kidney, under hypothermic conditions, may use lipids as the primary energy source. There is a high turnover rate of endogenous lipids, mainly triglycerides in the perfused kidneys, suggesting that energy supplementation is required.

One study by a San Francisco group has shown that the sodium-potassium pump activity in the cell membrane is markedly altered during perfusion and is coupled with a marked loss of intracellular potassium with time. Another

interesting observation made by this group is that the adenosine triphosphatase (ATPase) activity is directly related to the functional integrity of the cation transport system in the cell membrane. Their preliminary observations also indicate that the kidney under hypothermic conditions maintains a large percentage of its ATPase activity, which is considered to be directly correlated with successful preservation. Finally, these same authors have documented that there is intense vasoconstriction of the donor organ prior to removal in the agonal state. By the use of blocking agents, they and others have shown a marked improvement in survival. By using a preliminary perfusate in their perfusion apparatus, they are now able to predict which organ may have suffered irreparable damage prior to transplantation.

Basic Physiological Investigations

Support for studies of the basic mechanisms of kidney function, both in health and disease, continues to be an area of major research emphasis fostered through this program.

An area that has been receiving increased attention at the present time is the renin-angiotensin system. One group reports that isoproterenol, given intravenously, stimulates renin release at a much lower dose level than that which has been reported to be active when injected into the renal artery. The effect was found to be independent of renal hemodynamics and occurs after renal denervation, hypophysectomy, or adrenalectomy. This group suggests that a humoral mediator is responsible for the renin release although they do not totally discount the possibility of intrarenal shifts in circulation.

Another area which continues to receive a great deal of attention is uremia. Studies in a number of laboratories continue exploration of the events leading from normal renal function to advanced renal failure. A major focus of one group is on the adaptations in kidney function that attend the loss of functioning nephrons. They are approaching the adaptations both from the point of view of the alterations in the biologic control systems which subserve the maintenance of homeostasis, and from the cost to the organism for the adaptations. From their point of view one of the key elements in their studies of control systems is their sustained quest for a circulating inhibitor of sodium transport, which in their judgement may play a role in the regulation of sodium excretion not only in uremia but in health.

The role of peritubular physical factors in the control of renal proximal tubular fluid reabsorption has been well documented in recent years. There is convincing evidence for an effect of the oncotic pressure gradient across the peritubular capillary membrane. Other physical factors in the reabsorption process, such as the oncotic pressure gradient across the tubular wall and peritubular hydrostatic pressure gradients, although not fully clarified, are being investigated in several laboratories at the present time. One investigator is suggesting that the quantity, concentration and turnover of the interstitial albumin pool of the kidney may play an important role in the overall process of proximal tubular reabsorption. Thus far his experiments appear to suggest that in water diuresis a decreased concentration of the interstitial albumin pool alone does not provide a sufficient explanation for the decreased transit time of labelled albumin. This judgement is based on the observations that in two types of diuretic responses the increase in lymph flow and decreases in lymph

albumin concentration were similar, and that inulin mean transit times also shortened to a similar degree. In spite of this, only in the diuresis evoked by hypotonic fluid was the mean transit time of labelled albumin shortened. Thus the size of the interstitial albumin pool may have contracted in a similar manner in both saline and water diuresis, but in water diuresis the flux of albumin also increased to produce a large reduction in the mean transit. Although direct experimental measurements of the size of the interstitial albumin pool during saline and water diuresis are not available, this group expresses a view that a change of peritubular capillary permeability during water diuresis is the most probable explanation for their indirect findings.

Conferences

The fifth International Nephrology Congress will be held October 1972, in Mexico City. Both the NIAMD and NHLI have each awarded $25,000 from their fiscal year 1972 funds to support the Congress. These Congresses, which are held every three years, afford the opportunity for investigators, world-wide, who are engaged in nephrological research, to come together to exchange ideas and compare results.

Although the support for both a Urolithiasis and a Micropuncture Conference during the current fiscal year came from funds administered by the contract operation of this Institute, the Kidney Disease and Urology Program staff played a role in their preliminary planning. The Conference on Urolithiasis was initiated to bring together biological scientists and physical scientists, in an effort to set forth new ideas and encourage collaborative efforts which will hopefully promote progress in a field hampered by a lack of information about the causes of urolithiasis. The Micropuncture Conference was organized as an attempt to discuss and clarify a number of controversial issues in the area of micropuncture as applied to renal physiology and pathophysiology. Furthermore, aside from the exchange of ideas, the participants had the opportunity to discuss in-depth a number of the more important recent technical developments in this difficult and sophisticated approach to the study of kidney function.

Cooperative Studies

The Kidney Disease and Urology Program provides support for two long range studies of the nephrotic syndrome; one in adults and the other in children. The purpose of both studies is to test the therapeutic effectiveness of adrenal corticosteroids and cyclophosphamide as a treatment for this illness. Both are rather straight forward cooperative clinical trials of the effectiveness of the two drugs. As ancillary benefits, information will be gained on the correlation of the pathological and clinical manifestations of the disease, plus the establishment of a cooperative network of investigators.

New Support Instruments

An effort is now underway to adapt the presently utilized Academic Career Award and the Clinical Investigator Award of the Digestive Diseases and Nutrition Program, for the Kidney Disease and Urology Program. A recent survey has indicated that twelve percent of the academic urology positions are vacant and it is

anticipated that these awards will eventually help to alleviate that situation as well as the pressing need for more nephrologists. The target date for awarding a limited number of these awards is the spring of 1973.

M. James Scherbenske, Ph.D.
Kidney Disease and Urology Program Director

ANNUAL REPORT - TRAINING AND FELLOWSHIPS, NIAMD-EP

Introduction

Three recent developments are considered here:

1. Introduction of the Clinical Investigator and Academic Career Development Awards.

2. Assessment of the Institute's experimental group of training programs.

3. Change in RCDA review procedure.

The introduction of new support instruments, an inquiry into the usefulness of an existing program, the reallocation of responsibility for initial review for RCDA, these developments are considered here for their effects on the Institute's search for a more unified perspective on our diverse educational support endeavors.

The perspective sought here is one that looks beyond a mere listing of training grants, fellowships and career-type support to considerations of interrelationships and the utilization of these in decisions governing individual awards and relative program size. An appendix provided with last year's report gave a description of the relative contributions of Training Committees and Advisory Council in achieving such perspective. The events considered here point up the need for more effective interaction between them. The three developments considered all point to a distinctive role played by the initial review group that handles the Institute's RCDA applications. The shift of this responsibility to Study Sections may result in either the dissolution of a group that sees itself as no longer serving a purpose, or in its continued evolution as a needed unifying force in an organization whose function demands significantly compartmentalized endeavor.

The Clinical Investigator and Academic Career Development Awards

Other sections of this report treat substantive features of these awards in detail. We are concerned here with operational steps toward effective integration into the total Institute educational support effort. The Training Committee (Gastroenterology and Nutrition), working with Drs. Lionel Bernstein, George Kitzes and Sarah Kalser, took an active part in early program analysis leading to identification of need and the formulation of program guidelines. The Training Committee was thus well equipped to implement these in evaluating the applications received. The most difficult decisions were encountered, as expected, in arriving at appropriate weightings for the relative contribution to decisions on the Academic Career Development Award of individual qualifications on the one hand, and to institutional plans for development of the area, on the other. Differences of these two criteria served to bring out in Council discussion contrasting views as to how these awards should fit into the larger support pattern.

Council's role had begun with the discussion of concepts, and continued with review of successive drafts of guidelines. The general task of second-level

review and integration into the funding plan was facilitated by sufficient numbers of meritorious applications to make selection a significant process, and sufficient funds from an unexpected source (late release of fellowship funds previously withheld). The chief difficulty was the reluctance by some in accepting any criterion other than the applicant's accomplishments and potential. Considerations marshalled on both sides showed pronounced similarity to those evoked in the review of the RCDA, more specifically the renewal application for the outstanding performer, where the issue lay between those who would assign rank order on a simple scale of excellence and others favoring restriction of RCDA support to those in a demonstrably developmental career stage. The NAAMD Council plays no part in decisions on the RCDA award, but the presence on Council of one who had previously served on the RCDA Review Committee provided the tenuous but vital link. He pointed out the parallel between the two situations in a manner that paved the way for consensus.

The continuity of view provided by staff and xerox would have benefitted by something more direct, more personal, such as actual meeting and discussion involving representatives of Training Committee and Council. Given the profound uneasiness occasioned by the initial limitation of these new instruments to two of our eleven programs, the involvement of representatives from the several Institute Training Committees would have done much to keep the uneasiness realistic. Should the evolution of new support instruments call for continued restriction to only a part of our programs, and for funding at the expense of training grants, the need for more effective liaison is expected to become more pronounced.

The Institute's Experimental Group of Training Programs

Pursuant to plans noted in last year's report, this group of programs has been the object of assessment, both in relation to the categorical group and to the broad aim of fostering innovation in graduate medical education. The group convened for this purpose had a composition much like that suggested in the preceding section to deal with certain hurdles encountered in fitting the new support instruments into the larger pattern; in addition to the parent committee responsible for review (the same committee serves in the review of RCDA applications), the group included representatives from the several Training Committees and Council.

Briefly stated, the group looked forward to continuing benefit from deliberate channeling of some small fraction of our training support into training programs of other than the usual categorical postdoctoral sort. This easy generalization agreed upon, the hard work came in reckoning with attendant dilemmas:

- the disparity in time scales of our support and evaluation requirements, and the period required for significant developments in training investigators;

- the contrasting review problems in assessing "innovation" when untried, and five years later;

- the intensification of these problems as we analyze our notions of peer review, our dependence upon program comparison;

- the uncertain credentials of innovation as a recognizable characteristic of sustained programs rather than occasional student-preceptor or preceptor-preceptor encounters.

In considering the likely thrust of innovative effort, particular attention was given to programs comprising the training endeavor of several divisions and their usefulness to developing schools. Setting a term to programs currently supported was recommended as a means of fostering a start in this direction; setting a term to all support designated innovative, although acknowledged as desirable in some instances, was not acceptable as a general rule. Concerned here with the aim of achieving a more coherent approach to our educational support programs, stress is placed on the pivotal role in its achievement of appropriately interacting review and advisory groups. Success in fostering alternatives to the categorical approach in investigator training has depended upon translating their separateness of concept into something that operationally must inevitably be communicating. And this it has been, for the departments using both patterns of support, for the occasional faculty member participating in both types of programs, for the occasional student finding now one and then the other suited to the sequence of his developmental experience. In their administration, separation was achieved by assigning responsibility for conceptualization and review to a separate group convened for the purpose, making integration with the total effort the responsibility of staff and Advisory Council. Subsequent shift of initial review responsibility from an ad hoc group to the Research Career Program Committee established a significant connection with the categorical training programs at the initial review level as well, representatives of the latter participating with some regularity in the former's review work.

Implementing and testing of the direction emerging from this assessment lies ahead. In the continuing effort to work out a review structure that could guarantee some separation of innovative effort from the mainstream without complete isolation, the interaction of consultants is evolving in a way that could facilitate the handling of varied aspects of planning and review of our educational support.

Change in RCDA Review Procedure

It has been their responsibility for initial review of RCDA applications that has given the RCP Committee its identity. Shift of responsibility for initial RCDA review to the study section has prompted a year of exploration to determine whether some modified combination of responsibilities for training programs (experimental, multicategorical, in their initial review), new support instruments and RCDA's (second level of review) and Institute forum for planning and evaluation of educational support, could command the continued interest of this group. A major feature of this exploratory year has been a stepwise change in the handling by this group of the RCDA application. A summary of these steps is provided here.

The fall 1971 initial review was conducted by the study sections and Institute committee quite independently, the working out of a suggestion made by Dr. Bernstein and accepted with enthusiasm by the committee. Comparison of outcomes for the groups' 32 applications was undertaken at the winter meeting, a step considerably facilitated by a parallel tabulation prepared by Mrs. Zukel.

The winter group of applications was reviewed with results of study section review in hand, a step whose accomplishment owes much to the willingness of study section staff to make their results available ahead of their regular schedule. Now half-way into the transition from initial to second-level review, the group devoted a significant part of their effort to comparison of their initial intent with the net result of modification of study section recommendations, and further to the competing claims of application excellence and program balance. A continuation of this shift in emphasis is planned for the spring meeting, at which time the subject of Council liaison will be explored.

Summary

The three major developments comprising this report together point up the critical importance of effective communication, not only between initial and second-level review, but among the several initial review groups as well.

1. The launching of new support instruments presented an obvious problem in communication between initial and second-level review, as well as one less obvious but equally important, in conveying to the full range of Institute program groups the rationale of stepwise introduction of instruments serving a general need.

2. Inquiry into our accommodation of flexibility and innovation in training support and the appropriateness of a specially designated Institute program for this purpose was conducted utilizing a review group responsible for initial review of the experimental group now active, with additional representatives drawn from Training Committee and Council. A group of this composition could contribute much to unification of our planning and evaluation efforts.

3. The shift of initial review responsibility for the RCDA to the study sections enables the RCP Committee to explore a role in greater depth, starting with appraisal of their changing role in decision making on the RCDA's.

William H. Batchelor, M.D.
Training Grants Officer

Marjorie C. Zukel
Fellowships Officer

ANNUAL REPORT OF OPERATIONS BRANCH, NIAMD-EP

Administrative Services Section

Services provided by this Section include travel for both staff and consultants, personnel, time and leave, and a conglomeration of other administrative functions. During the past year, we have experienced the need to devote more time to the day-to-day projects. For instance, individual trip orders and vouchers have to be prepared for our travelers instead of fiscal orders and monthly vouchers; there is more concern and time expended in EEO matters, and nine employees are enrolled in Federal City College courses and upward mobility programs.

To do an acceptable job in all areas, more personnel are required. During the interim period, priorities will be established and those functions most essential to the successful operation of the organization will be performed.

Grants Operations Section

During the past year, the 15 percent increase in funds available for research grants and a smaller increase in fellowship funds occasioned a corresponding rise in the number of awards processed in the Grants Operations Section. The initiation of two new Programs, (the Clinical Investigator Award and the Academic Career Development Award in Digestive Diseases and Nutrition) increased the volume of work in the two units responsible for the processing of applications and the preparation of them for Committee and Council review.

There has been no increase in personnel to offset the additional workload. A major reorganization of the file room was undertaken in an effort to facilitate the handling of grant files and to conserve space. All members of the Section contributed to this effort whenever their work permitted.

Various employees of the Grants Operations Section are enrolled in the Adult Education Courses, the Basic Biology Course, the Federal City Upward Mobility College, and taking university courses at night in an effort to prepare themselves for the future.

Linden F. Neff
Grants Management Officer

ANNUAL REPORT OF GRANTS MANAGEMENT BRANCH, NIAMD-EP

This year a major innovation took place at the NIH affecting the management of grants. This changes involves the awarding of indirect costs. Until this year, it has been the direct responsibility of the Grants Management Branch to both calculate and award indirect costs associated with individual grants. Last July 1, this function was removed from the awarding components and placed in a newly formed organization, the Indirect Cost Management Section of OFM. Despite this change, it remains necessary for the GMB to prepare an additional document for each grant, detailing information as to the base to which the indirect cost rate applies and the location of the work. Further, since the ICMS award of indirect cost is taken from our appropriation, it continues to be necessary for us to calculate the indirect costs. This is required to verify the calculations under the new system, and to have information about encumbrances, both direct and indirect, at all times. Another facet of this new system involves the settlement of indirect cost claims for prior fiscal years. These claims must be met by using balances available from the fiscal year in which the award was made. There has been concern that sufficient balances will not exist for this fiscal year. Since unobligated balances in terminating projects is the major source of funds for this purpose, more time will have to pass before we can be positive that a problem exists. It appears that during the interim period some precautionary moves will be made.

Another major policy issuance concerns the accountability for equipment purchased with grant funds. This policy provides for the settlement of equipment accountability by one of several options at the conclusion of each project period. Recently, the Associate Director for Extramural Programs delegated authority to two members of GMB to approve waiver of accountability.

Typical of the trend toward increased concern for the use of research subjects (man and animal) is the recently issued policy for Protection of Animal Subjects. This was preceded by last year's issuance of a policy for Protection of Human Subjects. The latest policy for animal protection imposes still another burden on GMB for assuring, prior to each award involving animal subjects, that the grantee has filed an assurance with DRG concerning their treatment of such animals.

As of this writing no changes have occurred in the personnel make-up of the Branch. However, it is anticipated that one Grants Management Specialist will be leaving before June 30. This will leave the Branch again in the position of needing to recruit another specialist and/or fiscal management person.

Linden F. Neff
Grants Management Officer

ANNUAL REPORT OF ANALYSIS AND EVALUATION BRANCH, NIAMD-EP

For the second time in the Annual Report it is necessary to call attention to the fact that the Branch is still functioning without benefit of a full-time Chief. Last year the loss of a senior staff member whom we were unable to replace due to personnel restrictions was reported. Shortly after the beginning of the report period another valuable member of staff resigned. Her place was only partly taken by a more junior and part-time worker who has served us faithfully but whom we expect to depart also. Attempts are being made to secure another helper, even if at the lower levels, before this report year closes. Even so, present staff will still be unable to take care of peak workloads on a realistic schedule without overtime.

It is disappointing that the workload imposed by routine activities has infringed greatly on the preparation of special reports. Accordingly, we have not been as useful to some members of the EP staff as the Branch would have wished. This situation is quite likely to prevail until a Chief of the Branch is named.

In the Scientific Coding Section also there has been a decrease in personnel. For over half the year one of the staff, who previously functioned at NIH in minority group committee activities, was detailed to the Office of the Secretary. During this period no replacement could be secured. Following this detail she was selected for a position in the Equal Opportunity Program of the Department. With the personnel limitations prevailing we no longer have a full-time position available. At the present time we are desperately seeking a part-time person to help in the scientific coding and to permit us to stay ahead of the time demands imposed by the grants review and awarding cycles. While we are still hopeful that eventually our training grants and fellowships will be integrated with the research program data, this is obviously for the future when more heads and hands may be made available.

This Branch takes responsibility for the tabular material following this report showing the extent of NIAMD-grant operations for FY 1971 and the level now estimated for FY 1972.

Edward P. Offutt, Ph.D.
Acting Chief, Analysis and Evaluation Branch

TABLE I

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

Support of Research Grants

Program Area Number	Name	Actual July 1, 1970--June 30, 1971 No. Grants	$ Encumbered	Estimated July 1, 1971--June 30, 1972 No. Grants	$ Encumbered
01	Arthritis	153	8,097,813	143	8,780,000
02	Dermatology	45	2,088,494	55	2,901,000
03	Diabetes	121	4,982,353	111	5,688,000
04	Endocrinology	261	11,253,916	228	10,968,000
05	Digestive Diseases	206	9,179,784	217	11,207,000
06	Hematology	169	7,045,709	179	8,697,000
07	Metabolism	441	18,007,661	404	19,792,000
08	Nutrition	129	4,792,469	134	6,528,000
09	Orthopedics	73	3,735,173	84	4,871,000
10	Physical Biology & Related Areas	102	3,298,174	87	3,949,000
12	Kidney Disease & Urology	186	8,253,454	195	10,235,000
	Totals	1,886	$80,735,000	1,837	$93,616,000

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TABLE II

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

Support of Training Grants

Program Area Number	Name	Actual July 1, 1970--June 30, 1971 No. Grants	$ Encumbered	Estimated July 1, 1971--June 30, 1972 No. Grants	$ Encumbered
01	Arthritis	28	1,410,386	27	1,400,000
02	Dermatology	24	1,439,031	23	1,425,000
03	Diabetes	18	827,574	13	693,000
04	Endocrinology	41	2,132,215	41	2,310,000
05	Digestive Diseases	35	2,100,771	31	1,981,000
06	Hematology	36	2,081,149	32	2,006,000
07	Metabolism	27	1,343,328	21	1,233,000
08	Nutrition	12	601,682	8	558,000
09	Orthopedics	15	541,970	18	682,000
10	Physical Biology & Related Areas	6	897,968	5	883,000
12	Kidney Disease & Urology	35	1,695,926	39	1,901,000
	Totals	277	$15,072,000	258	$15,072,000

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TABLE III

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

Support of Regular Fellowships - F02

Program Area Number	Name	Actual July 1, 1970--June 30, 1971 No. Grants	$ Encumbered	Estimated July 1, 1971--June 30, 1972 No. Grants	$ Encumbered
01	Arthritis	8	65,884	4	31,000
02	Dermatology	4	36,631	3	27,000
03	Diabetes	16	140,782	12	89,000
04	Endocrinology	13	97,933	16	119,000
05	Digestive Diseases	4	40,247	8	60,000
06	Hematology	6	53,914	8	66,000
07	Metabolism	50	447,301	43	342,000
08	Nutrition	4	43,041	5	40,000
09	Orthopedics	4	32,400	4	30,000
10	Physical Biology & Related Areas	6	36,930	4	26,000
12	Kidney Disease & Urology	11	82,583	10	83,000
	Totals	126	$1,077,646	117	$913,000

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TABLE IV

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

Support of Special Fellowships - F03

Program Area Number	Name	Actual July 1, 1970--June 30, 1971 No. Grants	$ Encumbered	Estimated July 1, 1971--June 30, 1972 No. Grants	$ Encumbered
01	Arthritis	11	143,749	11	144,000
02	Dermatology	5	70,260	9	100,000
03	Diabetes	6	69,646	6	61,000
04	Endocrinology	12	161,990	20	233,000
05	Digestive Diseases	7	83,166	10	112,000
06	Hematology	9	122,676	7	104,000
07	Metabolism	11	125,841	7	89,000
08	Nutrition	1	18,950	2	26,000
09	Orthopedics	6	64,753	4	64,000
10	Physical Biology & Related Areas	0	1,000	1	9,000
12	Kidney Disease & Urology	28	364,891	15	184,000
	Totals	96	$1,226,922	92	$1,126,000

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TABLE V

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

Support of Research Career Development Awards - K03 or K04

Program Number	Area Name	Actual July 1, 1970--June 30, 1971 No. Grants	Actual July 1, 1970--June 30, 1971 $ Encumbered	Estimated July 1, 1971--June 30, 1972 No. Grants	Estimated July 1, 1971--June 30, 1972 $ Encumbered
01	Arthritis	11	261,865	12	270,000
02	Dermatology	4	95,476	5	120,000
03	Diabetes	11	242,663	13	292,000
04	Endocrinology	16	422,275	13	327,000
05	Digestive Diseases	16	410,907	17	403,000
06	Hematology	15	391,284	16	401,000
07	Metabolism	18	385,888	18	383,000
08	Nutrition	5	118,329	5	124,000
09	Orthopedics	4	25,000	2	45,000
10	Physical Biology & Related Areas	4	85,749	4	93,000
12	Kidney Disease & Urology	8	206,785	4	94,000
	Totals	112	$2,646,221	109	$2,552,000

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TABLE VI

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

Support of Research Career Awards - K06

Program Number	Area Name	Actual July 1, 1970--June 30, 1971 No. Grants	Actual July 1, 1970--June 30, 1971 $ Encumbered	Estimated July 1, 1971--June 30, 1972 No. Grants	Estimated July 1, 1971--June 30, 1972 $ Encumbered
01	Arthritis	2	58,487	2	59,000
02	Dermatology	0	0	0	0
03	Diabetes	1	30,915	1	31,000
04	Endocrinology	12	359,773	13	391,000
05	Digestive Diseases	0	0	0	0
06	Hematology	1	30,780	1	31,000
07	Metabolism	6	161,736	5	154,000
08	Nutrition	3	86,366	3	86,000
09	Orthopedics	0	0	0	0
10	Physical Biology & Related Areas	0	0	0	0
12	Kidney Disease & Urology	2	61,164	0	0
	Totals	27	$789,221	25	$752,000

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TABLE VII

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

Support of Academic Career Development Awards - K07

Program Number	Area Name	Actual July 1, 1970--June 30, 1971 No. Grants	Actual July 1, 1970--June 30, 1971 $ Encumbered	Estimated July 1, 1971--June 30, 1972 No. Grants	Estimated July 1, 1971--June 30, 1972 $ Encumbered
05	Digestive Diseases	Not active in other program areas nor in other years.		5	133,000
08	Nutrition			1	29,000
	Totals			6	$162,000

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TABLE VIII

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

Support of Clinical Investigator Awards - K08

Program Area Number	Name	Actual July 1, 1970--June 30, 1971 No. Grants	$ Encumbered	Estimated July 1, 1971--June 30, 1972 No. Grants	$ Encumbered
05	Digestive Diseases	Not active in other program areas nor in other years.		5	133,000
08	Nutrition			4	102,000
	Totals			9	$235,000

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OFFICE OF THE ASSOCIATE DIRECTOR FOR PROGRAM ANALYSIS
AND SCIENTIFIC COMMUNICATIONS

OFFICE OF SCIENTIFIC COMMUNICATIONS

Table of Contents

The Office of Scientific Communications is charged with the general programming and planning of communication activities concerned with the various scientific areas under the purview of the NIAMD. The present activities can be roughly divided into four main categories:

1. Current awareness literature services;
2. Retrospective literature studies;
3. Conferences and workshops; and
4. Other aspects of communication, such as the production of motion pictures, thesaurus development, etc.

Current awareness literature services are provided by the Institute in several specialized areas. There are three publications which provide subject organized listings of literature citations and one publication which expands to include abstracts.

ARTHRITIS AND RHEUMATIC DISEASES ABSTRACTS, the oldest of our publications, with the availability of Excerpta Medica's ARTHRITIS AND RHEUMATISM in this country, was no longer published after the sixth volume. GASTROENTEROLOGY ABSTRACTS AND CITATIONS, now in its sixth volume, is intended to provide abstracts and literature citations in all aspects of gastroenterology, biochemical or clinical.

The ARTIFICIAL KIDNEY BIBLIOGRAPHY (sixth volume), ENDOCRINOLOGY INDEX (fifth volume), and DIABETES LITERATURE INDEX (seventh volume), all contain citations to current world-wide literature which have been included by the National Library of Medicine in their Medical Literature Analysis and Retrieval System (MEDLARS). In order to serve those who are interested in highly specific areas, we have cooperated with the National Library of Medicine and four universities--Minnesota, Rochester, Case Western Reserve, and Vanderbilt--to produce structured, hierarchically-arranged indexes which function as in-depth current-awareness tools for easy review of particular subjects. The standardized terminology used in MEDLARS makes these publications invaluable for retrospective literature searching.

Conferences and workshops which are sponsored by NIAMD are planned and organized by the Scientific Communications Office. In addition to the organization, which usually includes physical arrangements as well as program planning with the conference chairman, the publication of the proceedings also falls under the auspices of the Scientific Communications Office. This year five conferences and three workshops were sponsored by the NIAMD. The conferences were the Fifth Annual Contractors' Conference of the Artificial Kidney-Chronic Uremia Program, Conference on Adequacy of Dialysis, Conference on Renal Micropuncture, Conference on Urolithiasis, and the Psoriasis Topical Chemotherapy Planning Conference; the workshops were the Workshop to Assess Increased Mortality in Middle-Aged Diabetics, Workshop in Blood Access Systems in Hemodialysis, and the Workshop on Cell Controls in Psoriasis. In addition, a series of meetings on assessment of the University Group Diabetes Program clinical trials of oral antidiabetic agents was initiated.

In addition, numerous other activities which bear on scientist-to-scientist communication as it affects the Institute are dealt with frequently. Areas where further emphasis should possibly be placed are continually in review.

The CBAC pilot project with DCRT in which the Scientific Communications Office (cooperating with Dr. J. E. Rall who was coordinating the effort) furnished a list of interested NIAMD Scientists to DCRT, helped construct profiles for these scientists, and designed an evaluation form, was evaluated to determine the responses of the NIAMD scientists. The pilot study indicated there was sufficient evidence of interest among the NIH scientists to offer the service to the NIH investigators.

Expansion of the Artificial Kidney Bibliography is underway to include other aspects of relevance to research on artificial kidneys such as kidney diseases and nephrology. An experimental edition was assembled and a meeting of the editorial advisory committee was held to discuss the experimental edition. MEDLARS searches are being designed to include the recommendations of the editorial advisory committee. Work with the International Committee for Nomenclature and Nosology of Renal Disease is progressing to include as much of the International Committee's nomenclature in the bibliography as feasible.

The Index of Dermatology was established and developed in cooperation with the National Library of Medicine over a period of three years by Universities Associated for Research and Education in Pathology under a grant from NLM. This grant expired in June, 1971, whereupon five dermatology societies jointly sponsored its publication until March, 1972. To further the NIAMD Dermatology Program as well as respond to the increased interest and activity in dermatology, NIAMD undertook the publishing of Index of Dermatology in April, 1972.

Project Title: Artificial Kidney Bibliography

Professional Personnel: Mrs. Billie Mackey
Dr. Keatha K. Krueger
Dr. L. C. Terry

Man Years: Total: 0.9
Professional: 0.6
Other: 0.3

Project Description:

The Artificial Kidney Bibliography, now in its sixth volume, is a quarterly recurring bibliography on kidney failure, artificial kidneys and kidney transplantation, which is being produced with the aid of National Library of Medicine's (Medical Literature Analysis and Retrieval System) by the Scientific Communications Section, NIAMD. It contains about 500 citations per issue with the citations arranged hierarchically to enable similar subjects to appear together. An author index is included with each issue.

The Artificial Kidney Bibliography is made available without cost to National Institutes of Health grantees or contractors who are involved with work related to artificial kidneys, as well as to other government agencies with responsibilities in the area, medical school and hospital libraries. In addition to the free distribution, it is for sale by the Superintendent of Documents, Government Printing Office at a nominal price.

An expansion of this bibliography is underway with plans to include other aspects of relevance to research on artificial kidneys, such as kidney diseases and nephrology. An experimental edition was assembled and a meeting of the editorial advisory committee was held to discuss the experimental edition. Further MEDLARS searches have been designed based upon the editorial advisory committee's recommendations. Work with the International Committee for Nomenclature and Nosology of Renal Disease is progressing to include the International Committee's nomenclature in the bibliography as much as possible.

Project Title: Diabetes Literature Index

Contract Number: PH43-67-663

Amount: $83,391 (May, 1972-June, 1973)

Contractor: University of Minnesota

Project Officer: Dr. Keatha K. Krueger

Other Professional Personnel: Mrs. Billie Mackey

Man Years: Total: .70
Professional: .32
Others: .38

Project Description:

Diabetes Literature Index, a monthly current-awareness publication of the NIAMD (now in its sixth volume) was brought about through the cooperative efforts of NIAMD, NLM, the American Diabetes Association, and three universities (University of Minnesota, University of Rochester, and Case Western Reserve University), as well as a number of individual scientists.

Each month MEDLARS magetic tapes, which contain all of the current biomedical literature citations used for the preparation of the NLM's Index Medicus, are used for identification of all diabetes-related bibliographic citations. Through 1970 the Diabetes Literature Index has consisted of a keyword in title index and an author index resulting from a computer printout. Starting in 1971, the magnetic tape version of these indexes (prepared at the University of Minnesota) is used to generate copy for offset printing by the Government Printing Office on their Linotron. There are now three formats consisting of a hierarchical subject index of core diabetes literature, an abbreviated keyword in title index of all diabetes-related literature, and an author index including index terms along with the citations (for the first author only). Printing the citations under a preconceived hierarchical structure, which groups related subjects, makes it easier for the user to scan his particular interests in one section.

Diabetes Literature Index is published monthly by the Office of Scientific Communication, NIAMD, and is available, free upon request, to qualified individuals with NIH grants or contracts who are working in the field of diabetes research, as well as to other government agencies with responsibilities in this area, medical school and hospital libraries. In addition to the free distribution of this publication, it is for sale at a nominal price by the Superintendent of Documents, Government Printing Office.

Project Title: Endocrinology Index

Professional Personnel: Dr. Keatha K. Krueger
Mrs. Billie Mackey

Man Years: Total: .20
Professional: .05
Others: .15

Project Description:

The Endocrinology Index, now in its fifth volume, is a bimonthly publication of the NIAMD, which is produced by the National Library of Medicine's Medical Literature Analysis and Retrieval System (MEDLARS). Each issue contains about 4,000 citations of the current world literature in endocrinology. The citations are grouped in eight categories (pituitary, thyroid, etc.) and each category has a preconceived hierarchical organization of subject headings. A list of the index terms assigned to the original article by the indexer appears in the author section. Author and subject indexes appear in each issue.

Liaison was continued with the National Library of Medicine's MEDLARS indexers, the Editorial Office of the Endocrine Society and the publisher, Lippincott, for two endocrinology journals, Endocrinology and Journal of Clinical Endocrinology and Metabolism to be indexed directly from manuscripts by NLM's indexers resulting in faster input of these journals into MEDLARS, and, consequently, into Endocrinology Index.

The Endocrinology Index is available without cost to NIH grantees and contractors in endocrinological work, and to medical libraries. It is also for sale at the Superintendent of Documents, U. S. Government Printing Office at a nominal price.

Project Title:	Gastroenterology Abstracts and Citations
Contract Number:	NIH 71-2071
Amount:	$59,850 (January, 1971 - April, 1972)
Contractor:	Scientific Literature Corporation
Project Officer:	Dr. Keatha K. Krueger
Other Professional Personnel:	Dr. George Craddock Dr. Eric J. Maybach Mrs. Billie Mackey
Man Years:	Total: 1.8 Professional: .85 Others: .95

Project Description:

In January 1966 the NIAMD began publication of a current-awareness journal for gastroenterologists. Gastroenterology Abstracts and Citations, now in its sixth volume, appears monthly. Each issue contains approximately 350 abstracts of the most significant gastroenterological literature published in the world and approximately 750 citations covering other relevant gastroenterological literature. The abstracts and citations are divided into three main categories, Pre-clinical Sciences, Diagnostic Procedures and Gastrointestinal Diseases. These categories are subdivided into more specific groupings. Each issue contains a subject and author index and these are cumulated annually.

Gastroenterology Abstracts and Citations is available, free upon request, to qualified investigators with NIH grants or contracts in the field of gastroenterology, as well as to other government agencies with responsibilities in this area, medical school and hospital libraries. In addition to the free distribution of this publication, it is for sale at a nominal price by the Superintendent of Documents, U. S. Government Printing Office.

Project Title: Index of Dermatology

Professional Personnel: Mrs. Billie Mackey
Dr. Keatha K. Krueger

Man Years: Total: 0.3
Professional: 0.2
Other: 0.1

Project Description:

The Index of Dermatology was established and developed in cooperation with the National Library of Medicine over a period of three years by Universities' Associated for Research and Education in Pathology under a grant from NLM. This grant expired in June, 1971, whereupon five dermatology societies jointly sponsored its publication until March, 1972. To further the NIAMD Dermatology Program as well as respond to the increased interest and activity in dermatology, NIAMD undertook the publishing of Index of Dermatology in April, 1972.

The Index of Dermatology is a monthly recurring bibliography on skin diseases, both general and specific, causative and therapeutic agents, and contains aspects such as immunology, mycology, virology, and oral disease. The Index is being produced with the aid of National Library of Medicine's MEDLARS (Medical Literature Analysis and Retrieval System) by the Scientific Communications Office, NIAMD. It contains about 1200 citations per issue with the citations arranged hierarchically to enable similar subjects to appear together. An author index is included with each issue.

The Index of Dermatology is made available without cost to National Institutes of Health grantees or contractors, who are involved in work related to dermatology, as well as to other government agencies with responsibilities in the area, medical school and hospital libraries. In addition to the free distribution, it is for sale by the Superintendent of Documents, Government Printing Office at a nominal fee.

Project Title: Artificial Kidney - Chronic Uremia Program
Fourth Annual Contractors' Conference Proceedings

Professional Personnel: Dr. Keatha K. Krueger

Man Years: Total: 0.1
Professional: 0
Others: 0.1

Project Description:

The Fourth Annual Contractors' Conference, held January 20-22, 1971, brought together about 150 contractors' key staff members with consultants to the Program and Institute staff. The three-day session involved extensive study of existing contract-funded research and development projects with emphasis on program progress and projected plans for the future.

The conferees, in both plenary sessions and intensive individual workshops, communicated research results in the main areas of the Artificial Kidney Program: 1) Hardware and Mass Transfer; 2) Cannulas, Membranes, and Nonthrombogenic Surfaces; and 3) Clinical and Metabolic Studies. A considerable body of detailed and pertinent scientific and technical information was exchanged between the top staff of 65 currently active research and development projects, the Program's consultants and its staff. It is anticipated that this and future conferences will significantly assist attainment of the Artificial Kidney Program's goals--optimum artificial kidney development, improved clinical methodology, and better patient rehabilitation.

The Proceedings of the Conference were compiled and given wide distribution to the community of scientists interested in the field of artificial kidney and chronic uremia.

Project Title: Artificial Kidney - Chronic Uremia Program
Fifth Annual Contractors' Conference and Proceedings

Professional Personnel: Dr. Robert J. Wineman
Dr. Thomas M. Valega
Dr. Keatha K. Krueger
Mrs. Billie Mackey

Man Years: Total: 0.4
Professional: 0.2
Others: 0.2

Project Description:

The Fifth Annual Contractors' Conference was held January 24-26, 1972. About 150 participants who were contractors and their staff consultants to the Artificial Kidney - Chronic Uremia Program, and Institute staff attended. The three-day session involved extensive study of existing contract-funded research and development projects with emphasis on intercommunication of program progress and projected plans for the future. The presentations were grouped in three sessions: Hardware, blood access, and clinical and metabolic studies. The conferees in both plenary sessions and intensive individual workshops, communicated research results in the main areas of the Artificial Kidney - Chronic Uremia Program: 1) Hardware, Membranes, and Nondialyzer modes of Therapy, 2) Blood Access and Nonthrombogenic Systems, and 3) Clinical and Metabolic Investigations. A considerable body of detailed and pertinent scientific and technical information was exchanged between the top staff of 62 currently active research and development projects, the Program's consultants and its staff. It is anticipated that this and future conferences will significantly assist attainment of the Artificial Kidney Program's goals--optimum artificial kidney development, improved clinical methodology, and better patient rehabilitation.

The Proceedings of the Conference will be published and distributed in June, 1972.

Project Title: Conference on Adequacy of Dialysis

Professional Personnel: Dr. George Craddock
Dr. Keatha K. Krueger

Man Years: Total: 0.12
Professional: 0.08
Others: 0.04

Project Description:

The purpose of the conference was to bring together a group of qualified and interested persons to discuss the problems of how to develop better criteria of adequate dialysis treatment. Its goal was to determine whether or not at the present level of knowledge as determined by a day of deliberation, it will be worthwhile to hold a workshop on this subject. The conclusions of this conference were that there were distinct areas in which adequacy criteria may be applicable and are certainly desirable. Such areas include specific evaluation with possible assays of peripheral neuropathy, behavior, uremia, and bone disease in uremic patients undergoing various modes of dialysis therapy.

Project Title: Conference on Renal Micropuncture

Contract Number: NIH 72-C-234

Amount: $15,625

Contractor: Yale University

Project Officer: Dr. Keatha K. Krueger

Man Years: Total: .04
Professional: 0.2
Others: .02

Project Description:

This conference was held November 18-19, 1972 at Yale University. The overall objective was a serious effort to clarify and straighten out a number of controversial issues in the area of micropuncture as applied to renal physiology and pathophysiology. There were also in-depth discussions of the most important recent technical developments in the field. Approximately 100 individuals from the United States and abroad participated in the conference. A comprehensive summary is being prepared by Dr. Giebisch, and will be published later through the Government Printing Office.

Project Title: Conference on Urolithiasis

Contract Number: 43-64-44 Task Order Number 55

Amount: $13,200

Contractor: National Academy of Sciences

Project Officer: Dr. Keatha K. Krueger

Man Years: Total: 0.02
Professional: 0.01
Others: 0.01

Project Description:

This conference was held August 30-31, 1971 at the Mayo Clinic in Rochester, New York. The purpose of the conference and the subsequent proceedings is to stimulate interest among investigators in scientific disciplines not well represented in the area, and to encourage collaborative work among urologists and other investigators doing research on the formation of urinary stones. The conference focused on physicochemical research in the field. Proceedings of this conference are to be published as a National Academy of Sciences' monograph.

Project Title: Psoriasis Topical Chemotherapy Planning Conference

Professional Personnel: Dr. Laurence Miller
Dr. Keatha K. Krueger

Man Years: Total: .02
Professional: .01
Others: .01

Project Description:

The Psoriasis Topical Chemotherapy Planning Conference was held April 18-19, 1972. The purpose of the Conference was to plan and design experimental methods of screening chemotherapeutic agents, particularly for clinical trials in patients. Five dermatologists and five pharmacologists gave presentations and a tentative experimental design was suggested with compounds chosen for consideration.

Project Title: Behavioral Bioassays in Uremia Workshop--
Proceedings

Professional Personnel: Dr. Keatha K. Krueger
Mrs. Billie Mackey

Man Years: Total: 0.8
Professional: 0.4
Others: 0.4

Project Description:

The Workshop on Behavioral Bioassays in Uremia was held on November 19, 1970 at NIH. The aim of the Worshop was to bring together experts from several disciplines who were interested in relating the knowledge of clinical and experimental psychology, pharmacology, neurology, and nephrology to the problem of behavioral bioassays in uremia, and to elucidate pathogenesis of behavior in uremia. Thirty-five investigators discussed available and potential behavioral bioassays of uremia.

The Proceedings were compiled and published with distribution to participants at the Fifth Annual Contractors' Conference of the Artificial Kidney - Chronic Uremia Program of the National Institute of Arthritis and Metabolic Diseases. It is on sale at the Superintendent of Documents, Government Printing Office.

Project Title:	Workshop to Assess Increased Mortality in Middle-Aged Diabetic Patients
Professional Personnel:	Dr. Keatha K. Krueger (for Dr. G. Donald Whedon)
Man Years:	Total: 0.08 Professional: 0.04 Others: 0.04

Project Description:

This one-day workshop meeting is concerned with the increased mortality in middle-aged diabetic patients. This meeting will be held June 19, 1972. The purpose of the meeting will be to evaluate present epidemiologic data and to design epidemiologic studies which might be either retrospective or prospective.

Project Title: The Use of Gastrointestinal Absorbents in Uremia Workshop Proceedings

Professional Personnel: Dr. Keatha K. Krueger
Mrs. Billie Mackey

Man Years: Total: 0.8
Professional: 0.4
Others: 0.4

Project Description:

The Workshop on the Use of Gastrointestinal Absorbents in Uremia was held on January 19, 1971 at NIH and was attended by twenty-four experts in the fields of nephrology, gastroenterology, chemistry, pharmacology, and engineering to study the problem of using gastrointestinal sorbents as a mode of therapy in uremia.

Four investigators currently holding contracts in the Artificial Kidney-Chronic Uremia Program described their research findings including: a system of microcapsules that were able to bind urea and ammonia in vitro, data (in vitro and in vivo) on microencapsulated and unencapsulated activated charcoal, and in vitro studies on the ability of dialdehyde starch to remove urea and ammonia. A low-volume sorbent-based dialysate regeneration system has been used for periods of six months without evidence of acute or chronic toxic effects. This dialysate regenerating system used one liter of dialysate which was recirculated through sorbent columns composed of zirconium phosphate, carbonated zirconium oxide, and activated charcoal. Demonstration of the effectiveness of these sorbent agents was unsuccessful when the agents were administered orally or rectally to either normal or "uremic" dogs. On the other hand, in vitro and in vivo data were presented that supported the effectiveness of oxidized starch administered orally for the removal of urea and ammonia. In addition, a presentation on the metabolism of urea and ammonia in the gut of normal and uremic individuals was given by a world authority in this area.

The considerable discussion identified areas where there was a scarcity of knowledge, and where further study is needed, which included: physiology of the gastrointestinal tract in uremia, whether toxic substances associated with uremia other than urea would be excreted into the gut, and the interaction of gastrointestinal fluid components and sorbents.

The Workshop, while not offering any concrete answers, was beneficial in delving into those areas that needed further study and in opening new avenues of investigation. Proceedings are being compiled and will be published in the near future.

Project Title: Workshop on Blood Access Systems in Hemodialysis

Professional Personnel: Dr. Eric Maybach
Dr. Keatha K. Krueger

Man Years: Total: 0.15
Professional: 0.1
Others: 0.05

Project Description:

This workshop was conducted to explore the current methods and future of blood access systems in hemodialysis. It consisted of two separate sessions, one on September 28, 1971 for the more practical aspects of the problem and included individuals actually running dialysis centers, physicians, nurses and dialysis technicians; a second session on November 3, 1971 was for the more theoretical research aspects, and included nephrologists and engineers. The discussions included patient acceptance of various blood access devices; general problems associated with blood access devices; specific experiences with specific devices; suggestions for investigation of the problem areas in blood access devices; and the likely future trends in blood access devices.
Dr. Maybach presented a report of the workshop at the Fifth Annual Contractors' Conference, and the transcript of his remarks will appear in the Proceedings of the Fifth Annual Contractors' Conference.

Project Title: Workshop on Cell Controls in Psoriasis

Professional Personnel: Dr. Laurence Miller
Dr. Keatha K. Krueger
Mrs. Billie Mackey

Man Years: Total: 0.2
Professional: 0.1
Others: 0.1

Project Description:

The Workshop on Cell Controls in Psoriasis was held October 19, 20, 21, 1971, and brought together 76 participants to give presentations and discuss cell controls and how these controls might have an effect on psoriasis. The purpose of the three-day session was to achieve the following goals: (1) Critical analysis of current information on psoriasis by workers within the field of dermatology and in fields that appear related to the problem of psoriasis. (2) Identification and intensive examination of areas of research that appear most relevant to further progress in understanding and control of psoriasis. (3) Recommendations for continued research in specific disciplines to provide a multilevel program of integrated research on psoriasis. It is anticipated that this workshop will significantly assist in identifying productive research and stimulating integrated research efforts on psoriasis.

The Proceedings of the Conference will be published and distributed in June, 1972.

Project Title: University Group Diabetes Program (UGDP) - Assessment of the Clinical Trials of Oral Antidiabetic Agents

Contract Number: 177139

Amount: $5,040

Contractor: Yale University

Project Officer: Dr. Keatha K. Krueger (for Dr. Thomas C. Chalmers and Dr. G. Donald Whedon)

Man Years: Total: 0.08
Professional: 0.04
Others: 0.04

Project Description:

A group of biostatisticians has been called together to initiate on evaluation of the clinical trials of oral antihyperglycemic agents with reference to their effects on morbidity and mortality in diabetic patients, with particular reference to the vascular complications. The validity and conclusions of the University Group Diabetes Program will be primarily assessed. This group is meeting May 17-19, 1972.

Project Title: CBAC Project with DCRT

Professional Personnel: Mrs. Billie Mackey
Dr. Keatha K. Krueger

Man Years: Total: .05
Professional: .05

Project Description:

The CBAC Pilot was initiated by DCRT which had acquired CBAC (Chemical-Biological Activities) computer tapes. Searchable data elements on tape are the abstract, title of article, authors, original journal reference, molecular formulae and Registry Numbers assigned by Chemical Abstracts for each chemical compound. The Scientific Communications Office (cooperating with Dr. J. E. Rall who coordinated the effort) furnished a list of interested NIAMD scientists to DCRT, helped construct profiles for these scientists, designed an evaluation form and helped evaluate the study. The pilot study indicated there was sufficient evidence of interest among the NIH scientists to offer the service to the NIH investigators.

OFFICE OF THE ASSOCIATE DIRECTOR FOR PROGRAM ANALYSIS
AND SCIENTIFIC COMMUNICATION

ARTIFICIAL KIDNEY - CHRONIC UREMIA PROGRAM

Summary Report

Introduction

The Annual Reports for the previous two years provide a detailed background description of the problem of end-stage kidney disease and elaborate on a relatively new therapeutic modality for its treatment, chronic intermittent hemodialysis. In addition, these reports give a detailed description of the planned and centrally directed contract program of research and development organized at the National Institute of Arthritis and Metabolic Diseases in 1966--the Artificial Kidney - Chronic Uremia Program -- which is aimed at development of improved and less expensive dialysis hardware and methodologies and at optimal rehabilitation of chronic dialysis patients. The Annual Report for 1968-1969 also has outlined in detail the problems of highest priority to which the Artificial Kidney - Chronic Uremia Program is addressing most of its efforts. This background material is not repeated here but the reader might wish to familiarize himself with it by perusing the previous reports.

Current Activities

The Artificial Kidney - Chronic Uremia Program endeavors to bring about improvements in artificial kidney apparatus and methodologies, to increase their effectiveness, to decrease their initial and operational cost, and to improve the rehabilitation of patients treated with chronic intermittent dialysis. Optimal development of new, better dialysis apparatus and methods is still hampered by incomplete knowledge concerning the specifics of the uremic syndrome. Hence, knowledge gained from research into the fundamental aspect of the toxic nature of uremia and basic and clinical studies into the long-term effects of uremia and of chronic intermittent dialysis are critical to arriving at a reasonable solution to the overall problem. Thus, the Program not only sponsors hardware improvement per se, but stimulates and supports a wide spectrum of research ranging from fundamental studies designed to clarify the toxic nature of uremia and clinical studies on the complications of chronic uremia and maintenance dialysis, to development of improved dialysis or apparatus. During the last year there has been an increasing emphasis on laboratory and clinical studies intended to bring about a greater understanding of the nature of uremia and its major complications.

At present about 65 carefully selected research and development projects are in existence, financed by the Institute's contract funds. These contracts were placed with universities, nonprofit research laboratories and industrial concerns, and constitute a broad-spectrum approach to the problems of highest priority. Several projects each are focused onto the problem areas mentioned above and to related investigations which promise to make the treatment of end-stage kidney disease less expensive and more effective, including the maintenance of a national registry of all patients who are currently being maintained with the aid of dialysis.

Accomplishments

In recent years, hemodialysis has become an established and fairly predictable maintenance therapy. A new generation of novel "hollow fiber" artificial kidneys is being developed and is being produced commercially. The technology of artificial kidneys has improved considerably in the last six years and there now exist a broad array of devices ranging from highly sophisticated, centrally controlled hospital center systems, to relatively simple self-contained home units.

Re-circulating Dialysate Machine. An entirely new concept for detoxification of the uremic patient has emerged with the development of specific adsorbents for metabolic wastes. A semi-portable artificial kidney machine, more convenient and less expensive to operate than previous models, is being developed based on principles elaborated in Program-supported sorbent research.

After 18 months of evaluation on animals and two years of clinical study at Cedars Sinai Hospital, Los Angeles, during which some of the patients on the machine were successfully treated for periods of a year or more, the machine is now being distributed to selected medical centers and hospitals for a broad clinical evaluation. With special training, patients will be able to use the apparatus in their own homes. It eliminates costly plumbing and water purification costs. The machine requires only about a half-gallon of the recirculating fluid needed to help cleanse the blood of impurities, instead of 80 to 100 gallons formerly necessary for a single dialysis treatment. As less water is used, tap water rather than specially demineralized (and expensive) water can be employed. Development can now be foreseen of artificial kidneys which are as portable as a large portable television set or a sewing machine, which can be used in any room of a home regardless of plumbing facilities, or which the patient can take with him in the back of his car when he goes on vacation.

Other Innovations. Other innovations, such as the simple and compact Dow hollow-fiber kidney, which has gone into mass production, and an "envelope" kidney, pre-sterilized for use with the conventional Kiil apparatus, have been reported in previous summaries. The Kiil dialyzer, itself, the time-honored work-horse of flat plate dialysis, is being significantly modified to use multiple point rather than longitudinal ridge supports, a change resulting in a marked increase in mass transfer. Thus it is possible to decrease the size of this dialyzer while retaining its somewhat long dialysis time or (and this is being done increasingly) to retain the original size but to decrease dialysis time to fit into a single nursing shift in the hospital. "Single shift Kiil dialysis", now a reality, represents a considerable saving in dialysis costs.

Single Needle Dialysis. A unique method of establishing simplified and pain-saving blood-vessel access has been reported by a research contractor of the Program from Germany, currently a visiting scientist at the University of Utah. Because of the clotting tendencies of plastic cannulas, surgically constructed subcutaneous fistulas (connections between an artery and a vein) are being used increasingly. They have a psychological and physical drawback; before dialysis two large-bore needles must be inserted through the skin into the enlarged venous portion of the fistula. For obvious reasons most patients are needle-shy.

During the last year this scientist has perfected a novel method of blood-vessel access which requires the placing of only one needle into the blood-stream. Through a simple valve device, this single needle alternately sucks blood from the patient and returns dialyzed blood to his bloodstream. Clinical trials of the new device have been very successful and patients using it have expressed a strong preference for it since it "saves a stick a day." If necessary the single needle access device can also be used in a regular large vein of a patient without the need for the surgical construction of a fistula.

Control of Secondary Hyperparathyroidism. A contractor of the Institute Artificial Kidney - Chronic Uremia Program and grant-aided investigators at the Mayo Foundation, Rochester, Minnesota, have demonstrated a method of medical control of secondary hyperparathyroidism during long-term hemodialysis. Secondary hyperparathyroidism, which results from excessive secretion of parathyroid hormone (PTH), and renal osteodystrophy (dissolution of bone) are seemingly inevitable complications of chronic renal failure. It has often been suggested that subtotal parathyroidectomy may be desirable in all such patients undergoing long-term hemodialysis. The present studies were performed to determine whether a medical alternative to such surgery might be feasible.

Trials in 10 patients indicate that excessive PTH secretion can be significantly decreased by simultaneously decreasing plasma phosphate and increasing dialysate calcium. The investigators recommend use of their regimen in patients undergoing long-term hemodialysis.

Transketolase Inhibition in Uremic Damage to the Nervous System. At the New York Medical College, a contract from the Artificial Kidney - Chronic Uremia Program has enabled investigators to show that the plasma and cerebrospinal fluid of patients with chronic uremia who also have peripheral nerve damage contain a dialyzable toxic factor that inhibits activity of the enzyme transketolase in nerve tissue. The nature of the inhibitory factor is as yet unknown. This finding could explain the neurological damage characteristic of chronic uremia.

This damage is remarkably similar to that associated with myelin sheath degeneration seen in thiamine deficiency. The neurological damage associated with the latter disorder has been attributed to deficiency of an enzyme of the "hexose monophosphate shunt" biochemical pathway of carbohydrate metabolism, transketolase, which apparently is necessary for the maintenance of myelin and of its coenzyme thiamine pyrophosphate (TPP).

Investigation of transketolase activity (TKA) in patients with chronic uremia now has shown that TKA is significantly inhibited by the plasma and cerebrospinal fluid of such patients, and that chronic dialysis simultaneously removes the toxic substance, abolishes TKA inhibition and ameliorates the neurologic damage. Such studies may lead to isolation of a specific nerve toxin operative in uremic neurologic damage, as well as to a means of testing the clinical efficacy of hemodialysis.

Peritoneal Home Dialysis. Peritoneal dialysis is a method of filtering impurities from the blood which utilizes the lining of the abdominal cavity, or peritoneum, as a dialyzing membrane. In 1968, Tenckhoff and associates,

working with support from the NIAMD's Artificial Kidney - Chronic Uremia Program, developed an indwelling silicone rubber catheter which permitted safe access to the peritoneal cavity for repeated dialyses of patients in chronic (as well as acute) renal failure. Prior to this development it was necessary to puncture the abdominal wall with a trocar every time a peritoneal dialysis was contemplated, a circumstance which made routine, continually repeated peritoneal dialysis largely impossible.

Use of peritoneal dialysis with the aid of this catheter has now become a safe routine procedure and has permitted the introduction of this method of dialysis into the home. It has an important advantage, since it only requires one person for safe conduct of the dialysis--in contrast to hemodialysis which is not permitted in the home unless one other knowledgeable person is available. Also, peritoneal dialysis is the only resort for those patients in whom all available blood access sites have been exhausted.

An important new development has recently been introduced by this contractor, namely the preparation of pyrogen-free, sterile dialysate solution for peritoneal use with the aid of a new process--reverse osmosis. Several months of experience attest to the safety and usefulness of this relatively simple and inexpensive method of dialysate preparation which contrasts markedly with the great expense connected with the use of conventional commercially prepared intravenous solutions.

Future Activities of the Artificial Kidney Program

The Program will continue promising current research and development efforts and will pursue new ideas and approaches which appear feasible. The Institute is currently funding a number of studies aimed at the isolation and identification of unknown toxic factors which must be removed from the patient's blood, as well as of metabolic wastes and other substances known to accumulate during kidney failure, such as urea, creatinine, uric acid, potassium, hydrogen ion, and water. Much of the future success in simplifying the treatment of patients with end-stage kidney disease will depend on developments in this area. The Program also intends to follow very closely clinical and technological developments abroad and, when promising, to enlarge upon them for the benefit of United States patients as well. This is particularly pertinent since developments in several Western European countries have been quite promising and their early and active introduction in the United States and possible refinement (without waiting for the usual delay connected with spontaneous spread of new techniques) could prove highly beneficial to patients in the United States. For instance, the Institute has adapted and helped to spread information about a special "selected low-protein" diet, first developed in Italy, that can delay the terminal phase of kidney failure in many cases; the new "single needle" blood access, the idea for which originated in Germany, is an even more recent example.

Conferences, Publications, and Scientific Communication

The Institute regularly organizes symposia and workshops on critical problems or opportunities in kidney disease research. In September and November 1971, the Institute sponsored workshops on Blood Access Devices in Dialysis to

discuss current methods of connecting the bloodstreams of patients to artificial kidneys, recurrent problems, and ways to counter them. A two-day Renal Micropuncture Workshop was held in November 1971, at Yale University, with partial support from the Institute. The meeting helped partially to resolve problems of interpretation experienced in the relatively new, widely-used method of renal micropuncture, which permits examination and study of the kidney directly at the basic anatomical level of the single nephron.

In January 1972, the fifth annual review meeting of the Institute's Artificial Kidney - Chronic Uremia Program contractors brought together about 200 contractors' key staff members with consultants to the Program and Institute staff. The three-day session involved extensive study of existing contract-funded research and development projects with emphasis on program progress and projected plans for the future. A considerable body of detailed and pertinent scientific and technical information was exchanged between the top staff of the currently active research and development projects, the Programs's consultants and its staff. As in previous years, this material will be published in the form of concise Proceedings to be widely distributed to workers in the relevant fields. The Proceedings of the previous annual conferences have become definitive publications on the state of the art in dialysis and it is anticipated that this and future such conferences will significantly assist attainment of the Artificial Kidney - Chronic Uremia Program's goals: optimal artificial kidney development, improved clinical methodology, and better patient rehabilitation.

In April 1972, the Program organized a one-day interdisciplinary workshop on the topic of "Adequacy of Hemodialysis" under the chairmanship of Dr. Belding Scribner of the University of Washington. This workshop is being looked upon as the first of possibly several such efforts to define the current state of the art in terms of maximal rehabilitation of dialysis patients. Considering that about 5,500 patients are now maintained in the United States with the aid of dialysis, it has become an urgent necessity to define as closely as possible in quantitative terms of time, equipment and methodology used, what is adequate and what is marginal or inadequate dialysis treatment, and it is the hope of the Program to be able to sponsor a successful effort in this direction.

In the spring of 1972, the Program published a monograph "Evaluation of Hemodialyzers" which defines the data base required for evaluation of hemodialyzers from the early development phases to the final phase of long-term patient therapy. This publication 1) provides an overall view of the presently conceived requirements for a clinically useful dialyzer, 2) outlines the measurements essential for realistic evaluation of the device, and 3) provides references to the appropriate literature. It is the fruit of labor of an interdisciplinary group with representation in clinical nephrology, chemical engineering, and biomaterials toxicology, under the chairmanship of Dr. Frank Gotch of the University of California in San Francisco. This valuable monograph will be distributed to all members of the American Society for Artificial Internal Organs and the American Society of Nephrology.

Kidney Disease and Nephrology Index. The Institute has prepared an experimental, pilot issue of a proposed new bimonthly publication, Kidney Disease and Nephrology Index. This publication would list, through bibliographic citations,

every scientific paper related to kidney disease, which appears in the United States or anywhere in the world and is available through the National Library of Medicine's MEDLARS system. In this fashion, all interested research workers can be promptly alerted to the latest findings and developing knowledge related to kidney disease. Formal permission is required from the Office of Management and Budget to issue such a new publication regularly. Meanwhile, the experimental issue will be the subject of critical review by a board of editorial advisors, authorities in the kidney field.

Pamphlet on Kidney Disease and the Artificial Kidney. The Institute maintains an active program of public information. As part of this activity a new pamphlet Kidney Disease and Artificial Kidneys has been published. The brochure describes in simple terms how natural kidneys function, the diseases that affect them, and symptoms that provide danger signals. Artificial kidney treatment methods and transplant procedures also are outlined.

Outlook for Kidney Disease and Research and Development

Research conducted and supported by the National Institute of Arthritis and Metabolic Diseases has added much to the understanding and treatment of kidney disease. Investigations such as those discussed in this report have increased our ability to cope with the problems of kidney failure. Additional new research efforts are called for in order that a more widespread and permanent control of kidney disease can be achieved. A meaningful program to reduce kidney disease must include all of the various components -- research, prevention, treatment, education -- available in our armamentarium. Such a total program must be aimed at all primary kidney diseases as well as at end-stage kidney failure. This combination, led by much-needed research, should yield maximal benefits in the number of lives saved today and the deaths prevented in the years to come.

Contractor: Abcor, Inc.

Amount: $148,900

Title: Improved Cannula Materials for Hemodialysis

Objectives:
This contract is designed to develop and explore potential synthetic materials which will produce an artificial vascular intima and then fabricate a blood compatible hemodialysis cannula system from these materials.

Major Findings:
Work is continuing on the development of artificial intimas. Electrodeposited materials are being utilized in an attempt to make an extremely thin artificial vascular intima.

This contract will terminate this year.

Contractor: Abcor, Inc.

Amount: $88,433

Title: Development of Capillary Membrane Dialyzer

Objectives:

To develop an improved artificial kidney utilizing capillary membranes as the dialyzing surface and to clinically test this device.

Major Findings:

Because of high cost of construction and poor performance at low dialysate flows a new dialyzer design was created. This is a baffled cross flow dialyzer of square configuration with baffles running longitudinally and projecting alternately from the top and bottom surfaces of the shell. Performance data indicate a clearance of about 140 ml/min for urea and 90 ml/min for creatinine at a test solution flow rate of 200 ml/min. The same conditions result in a clearance of about 65 ml/min for uric acid and 10 ml/min for raffinose. Ultrafiltration has been lower than desirable; but work is planned to improve this factor.

Proposed Course:

This program is continuing in an effort to obtain *in vivo* data on animals and clinical data.

Contractor: Albany Medical College of Union University

Amount: $36,765

Title: Electronic EEG Frequency Analysis for Evaluation of Uremia

Objectives: The contractor's objective is to attempt to devise an electronic system which would enable detailed and sophisticated analyses of the EEG in uremia and to attempt to use it as a tool to predict the severity of the clinical status of the patient.

Major Findings: The program has developed the proposed electronic system and has demonstrated that even well dialyzed patients have abnormally slow EEGs when studied in this technique. However, in transplanted patients the abnormal slowing of the EEG was resolved in the first week after successful transplant.

Proposed Course: The investigator plans to apply this technique on patients at other centers to confirm the results he has obtained. Should the results be confirmed, then he expects to simplify the machine so it can be used as a way of judging the clinical state of the patient and the success of therapy.

In addition, he will study why the EEG of some dialyzed patients returns to normal while in others it does not.

Contractor: American Hospital Supply

Amount: $85,800

Title: Improved Cannula Development

Objectives:
This is a contract to develop an improved cannula with a flare tip design on the venous side. In addition an artificial type of sleeve pin design will be fabricated and tested in vivo.

Major Findings:
Preliminary flow studies indicated that a continuously slowly expanding tip of approximately 6 to 8 degrees taper seems to give a better flow in the cannula than does a more sharply divergent tip. Cannulas of various tip configurations have been developed and fabricated. A subcontract with the University of Washington is in the process of being negotiated for in vivo testing of these tip designs.

Proposed Course:
There will be in vivo evaluation of the already fabricated venous tips and based on the results of these in vivo tests further development of cannula tips will be done.

Contractor: Amicon Corporation

Amount: $43,490

Title: An Improved Hollow-Fiber Hemodialyzer

Objectives: The new contractor will continue to develop and evaluate a new hollow-fiber hemodialyzer. They will share the *in vitro* and animal testing with Cutter Laboratories as a subcontractor. It should be ready for clinical trials at the end of one year.

Major Findings: The contractor has successfully carried out *in vivo* and *in vitro* tests the $0.4M^2$ unit. The data indicates that the permeability characteristics of this Hollow Fiber Dialyzer are superior to currently existing hemodialyzers. There appears to be no problems with thrombosis.

Proposal Course: Amicon will continue the engineering development of the unit and scale up to produce a $1.0M^2$ unit for clinical testing. The subcontractor, Cutter Labs., will continue the *in vivo* evaluation and toxicity testing.

Contractor: Avco Everett Company

Amount: $109,801

Title: Development of Avcothane Cannulas

Objective:
Utilizing the proprietary substance Avcothane, these people proposed to develop a cannula of this Avcothane as well as to investigate various causes for recurrent thrombosis with the use of cannulas in hemodialysis.

Major Findings:
Avcothane cannulae have not yet been inserted in sheep. However, the principal investigator has discovered that the current silastic cannulae are quite variable in their lifetimes and is attempting to establish a a reliable baseline against which to measure the performance of the Avcothane cannulae.

Contractor: Avco Everett Research Laboratory

Amount: $97,981

Title: Blood Flow Considerations to Minimize Thrombosis in Artificial Kidney Systems

Objectives:

Investigation of hydromechanical phenomena and surface materials properties relevant to initiation and propagation of thrombus formation.

Findings:

The formation of thrombus in the separated flow region has been found to vary with the shear rate of the particular test chamber. Aggregation and subsequent thrombus formation occur more rapidly as the shear rate of the particular chamber increases. This effect scales in the opposite direction to results obtained in the simple stagnation point flow previously studied. Experiments have been continued in order to further define and understand the influence of a separated flow region on thrombus formation.

Experiments have also been completed to investigate thrombus formation in a tube flow configuration. These experiments have indicated the importance of properly controlling the entrance configuration to the tube. As a result of these experiments a new entrance configuration has been designed and will be employed.

Proposed Course:

1. Investigate blood/surface interfacial phenomena under controlled flow conditions as a function of systematically controlled surface properties of polyurethane test surfaces and systemic anticoagulation.

2. Investigate the influence of locally infused thrombus inhibiting agents as a means of controlling or eliminating thrombus formation in shunts or dialyzers.

Contractor: Battelle Memorial Institute

Amount: $118,000

Title: Feasibility of Microencapsulated Detoxicants for Removal of Metabolites Via Ingestion

Objectives:

The overall objectives of this contract are the development of microcapsules capable of irreversibly binding urea, ammonia and other toxic metabolites and the evaluation of these capsules for gut dialysis.

Major Findings:

The Battelle group have found that chemical binders of urea are inadequate and the most rewarding area of investigation is to develop methods to stabilize urease and bind this enzyme to polymers which can then be encapsulated. The urea would then react with the urease and aspartase and be converted to ammonia which then could be adsorbed by resins containing sulfonic acid groups which are also encapsulated. The stabilized urease has been encapsulated as well as the resins containing sulfonic acid. Their microencapsulated system has shown to be effective in vitro and in azotemic animals. The concentrations of selective nitrogenous metabolites in various regions of the dog small intestine were determined in control and azotemic animals and correlated to blood levels of creatinine urea, and uric acid.

Proposed Course:

They are presently planning to test their microcapsules in animals. They plan to study the physiology of the GI fluids and the behavior of these fluids with their microcapsules.

Contractor: Beth Israel Hospital

Amount: $106,845

Title: The Role of Guanidine Compounds in the Pathogenesis of the Uremic Syndrome

Objectives:

The investigator will quantitatively determine the levels of guanidine and the disubstituted guanidines in body fluids in patients with chronic renal disease.

Major Finding:

The contractor has developed reliable techniques for the isolation and quantitation of all the guanidines. He has determined that methylguanidine is not elevated in uremic serum.

Proposed Work:

He will develop a system to separate and quantify phenol and all its derivatives and determine if they are elevated in uremic serum.

Contractor: Peter Bent Brigham Hospital

Amount: $28,856

Title: Changes in Intellectual Ability and Performance Associated with Uremia and its Modification

Objective:

This is a contract to study the effects of uremia on cognitive, perceptual and memory functions, and modification by dialysis and transplantation. The investigator will administer and study the Continuous Performance Test and the Flicker Fusion Test, and other quantitative repeatable tests found suitable for evaluation of sustained attention, perception, motor coordination, response readiness and memory.

Major Findings:

Uremic patients perform poorly on the CPT. When dialyzed, they show improvement but do not return to normal, as do successfully renal transplanted patients.

Proposed Course:

To continue development of the CPT as a measure of efficacy of dialysis and a potential human behavioral bioassay.

Contractor: The Bronx-Lebanon Hospital Center

Amount: $56,400

Title: The Origin and Effect of Guanidinosuccinic Acidemia in Uremia

Objectives:

The contractor will investigate the quantitative interrelationships of guanidino and urea precursors in blood and urine samples of uremic patients who have had their diets altered with specific amino acids and guanidine precursors.

Major Findings:

The investigator has shown that guanidinosuccinic acid (GSA) is elevated in uremia, removed by dialysis and increased in normals who are fed a high protein diet. Work has also demonstrated that GSA induces a reversible thrombocytopathy which is correlated with changes in electron micrographs. He has recently noted that GSA levels are increased in animals when various urea precursors are fed to them but decreased when methionine is given. These findings have been reproduced in humans.

Proposed Course:

Terminated

Contractor: Brooklyn Veterans Administration Hospital

Amount: $12,000

Title: Study of Identity and Behavior of Proteins Adsorbed Out of Plasma onto Artificial Kidney Membrane Materials

Objective:
To find a correlation between the thrombogenicity of biomaterials intended for use as artificial kidney membranes, and the identity and behavior of plasma proteins adsorbed at the blood/material interface.

Major Findings:
1) Clotting times of intact plasma placed on single layer ultrathin cellulose membrane (SUCM) surfaces were significantly longer than those on prewetted glass.

2) Pre-exposure of SUCM and of glass surfaces to normal plasma, followed by thorough rinses, rendered the membranes less able to correct the PTT of factor XII deficient plasma than were the control surfaces.

3) Residues left by plasma residing for 12 seconds or more on prewet SUCM did not interact with anti-human serum or with anti-human fibrinogen, as indicated by water vapor condensation patterns developed subsequently.

4) Air interfaces on dry surfaces such as dry SUCM caused platelets to adhere where a drop of diluted plasma had been run over dried SUCM, and also, in other experiments, to an area where fibrinogen had been deposited at an air/plasma/stearate interface.

Proposed Course:
Studies of the effects of well-controlled flow upon the identity of proteins deposited in anodized tantalum foil tubes will be undertaken.

Study of 20/80 PEG,6000Copolycarbonate (PEG) films which are hydrophobic and of their effects after deposition on nonwettable silicon crystal slices caused by the flow of a bulk of plasma, and of fibrinogen.

Contractor: The Brooklyn-Cumberland Medical Center

Amount: $38,000

Title: The Effect of Peritoneal Dialysis and Hemodialysis on the Level of Water Soluble and Fat Soluble Vitamins in the Blood of Uremic Patients

Objectives:

To study the effect of hemodialysis and peritoneal dialysis on the level of water soluble vitamins in the blood of uremic patients to ascertain the specific vitamins removed by dialysis, to measure the amount of depletion of body stores, and to assess the significance of the losses. In addition, they wish to make specific suggestions regarding vitamin supplementation required in these patients.

Major Finding:

Patients who did not receive vitamin C supplements demonstrated a consistent decrease in plasma ascorbic acid during each dialysis with a mean decline of 40 $\pm$ from the predialysis value. During the course of the study two patients demonstrated skin changes consistent with those that occur in ascorbic acid deficiency.

Proposed Course:

Terminated

Contractor: University of California, San Francisco Medical Center

Amount: $8,000

Title: Clinical Evaluation of the Western Gear High Performance Multiple Point Support Dialyzer and Comparison to the Kiil Dialyzer

Objective: The *in vivo* transport characteristics of dialyzers have been generally defined by clearances of urea, creatinine, phosphate and uric acid. Equivalent clinical therapy in terms of dialysis hours has in practice, often been based on the ratio of urea or creatinine clearance between two dialyzers. Western Gear D_4 Multiple Point Support (MPS) dialyzers (developed under contract to the Artificial Kidney - Chronic Uremia Program of this Institute) were found to have creatinine and urea clearance ratios of 1.5 compared to Standard Kiils. A 33% reduction in MPS dialysis hours should provide equivalent clinical therapy if the above contention is true. However, if larger molecular weight toxic molecule clearances are key factors in dialysis therapy effectiveness, reduced treatment hours with the MPS might be expected to result in decreased net clearance of these solutes and possibly clinical deterioration. Net clearance of larger solutes is membrane area, permeability, and treatment time, dependent and it was initially assumed that these clearances would be equal in these two nominal 1M2 dialyzers. A study was undertaken to do the following:

1. Determine if a consistent, reliable and clinically useful relationship exists between dialyzer creatinine clearance, treatment hours and resultant patient well being.

2. Determine if dialyzer solute transport properties can be effectively used to study the influence of larger molecular weight solutes on the uremic symptomatology of dialysis patients. Specifically, whether decreasing the magnitude of larger molecular weight solute clearance while holding smaller molecular weight solute clearance constant will result in a detectable clinical change.

Major Findings:

1. Six patients underwent two to thirteen months of MPS dialysis with hours reduced in inverse proportions to the ratio of their MPS/Kiil *in vivo* creatinine clearances.

2. No deterioration in any clinical parameters specifically ascribable to reduced dialysis hours could be noted in these patients.

3. Study of aqueous Vitamin B_{12} and inulin clearance revealed the MPS to be 40-50% more efficient than the Kiil for these larger solutes. Consequently, the net clearance provided by the two treatment schedules was equal over the entire molecular weight range studied. Because of

this, no conclusions can be drawn regarding the influence of larger molecular weight solutes on the uremic symptomatology of dialysis patients.

Proposed Course: To continue this investigation for the completion of experimental data.

Contractor: University of California

Amount: $159,000

Title: Evaluation of Low Protein Diet in Dialysate

Objectives:

The contractor seeks to examine the effect of low protein diets in uremia in both dialyzed and nondialyzed patients, and to evaluate the optimal protein intake of patients on hemodialysis. He is also studying the biochemical phenomenon of protein metabolism in uremia.

Major Finding:

Studies were continued on low protein diets; work was done on the combination of low protein diets with infrequent dialysis. Patients received dialysis only once per eleven days and did well when utilizing a 26 gram protein diet obtained by the electrodialysis of whey. Erythrocyte transketolase levels have been found to be normal or elevated in the chronic uremic. Histidine has been demonstrated to be an essential amino acid for uremic patients.

Proposed Work:

The group will continue its study of amino acid metabolism in the chronically dialyzed patient. They plan a detailed study of tyrosine metabolism in uremia.

Contractor: Case Western Reserve University

Amount: $81,197

Title: Removal of Waste Metabolites from Dialyzing Fluid by Micro-encapsulated Reactants

Objectives:

To develop a new technique for removing waste metabolites from dialyzing fluid by encapsulating specific reactants inside hollow polymeric spheres.

Major Findings:

The investigators have successfully developed techniques for encapsulating solids and liquids. Studies of Giordano oxystarch confirm its previously reported high affinity for urea and/or ammonia.

Proposed Course:

Continued *in vitro* studies of encapsulation techniques and characterization of encapsulated materials.

Contractor: Case Western Reserve University

Amount: $58,121

Title: Application of Laser Doppler Techniques to Artificial Kidney Design

Objectives:

To apply the laser doppler flowmeter to: (1) kinetics of blood coagulation and (2) mass transfer through boundary layers adjacent to surfaces.

Major Finding:

1. Derivation of general relationship between the space - time correlation function and the observed spectrum from the laser doppler velocimeter.

2. In situ measurements of rate of formation of fibrin from fibrinogen.

3. Development and testing of techniques for determining degree of polydispersity of an arbitrary dispersion from the laser doppler signal.

Proposed Course:

1. Develop and check computational techniques which relate flow patterns near membranes to mass transfer rates.

2. In situ measurement of fibrinogen formation and platelet agglutination.

3. Develop techniques for experimentally determining degree of polydispersity within the flowing medium.

Contractor: Cedars-Sinai Medical Center

Amount: $152,033

Title: Osteodystrophy and Divalent Ions in Kidney Failure

Objectives: Perform a systematic and comprehensive study to evaluate the mechanism leading to deranged divalent ion metabolism in renal failure.

Major Findings: Evaluations of mechanisms involved in the development of abnormalities of divalent ion metabolism in 1) stable chronic renal failure patients; 2) patients undergoing chronic hemodialysis; 3) patients from post-renal homotransplantation; and 4) patients with early minimal degrees of renal failure in approximately twenty patients per group. Investigation into the role of Vitamin D resistance and altered parathormone activity in producing the skeletal and soft tissue pathology indicates that a progressive Vitamin D resistance occurs and skeletal unresponsiveness to parathormone in conjunction either initiates or aggravates the osseous and soft tissue abnormalities.

In addition the investigators have found higher levels of skin calcium in patients dialyzed with higher dialysate calcium concentrations, although phosphate intake was uncontrolled.

Proposed Course: Continue investigations into the mechanisms of divalent ion metabolism in uremic osteodystrophy with application of findings toward rational modes of therapy for this disorder.

Contractor: Cedars-Sinai Medical Center

Amount: $134,650

Title: Gastrointestinal Use of Sorbents

Objectives:

Determine the feasibility of using adsorbents in the gastrointestinal tract to treat patients suffering from chronic uremia. The adsorbents will be used in an attempt to decrease the urea load on the kidneys by enzymatic breakdown of the urea and adsorption of the products of this reaction within the gastrointestinal tract. An added dividend of this work may be its application for therapy of excessive serum and gut ammonia levels as found in hepatic failure cases.

Major Finding:

Initial studies of giving uremic dogs zirconium phosphate and activated carbon were not successful in lowering their BUN. Now they have developed extensive data on the level of constituents in the GI tract in a normal dog.

Proposed Work:

Studies on the physiology of the GI fluids in uremic and normal dogs and patients will be undertaken. The behavior of the sorbent with the GI fluids will also be studied.

Contractor: Cleveland Clinic

Amount: $40,000

Title: Development of an Envelope Kidney

Objectives:
1) To develop an envelope (presterilized) membrane for use in a Kiil;
2) to develop a coil envelope (low cost, convenient, presterilized); and
3) to evaluate the feasibility of an intracorporeal artificial kidney.

Major Findings:
A Kiil-Envelope has been successfully developed and clinically tested and is ready for marketing to the public. The second objective is being developed along lines similar to #1. The intracorporeal kidney was not found to be feasible.

Proposed Course:
Objective (2)

Further evaluation of the intracorporeal kidney will not be performed.

Contractor: Columbia University

Amount: $169,700

Title: Identification of a Nonthrombogenic Environment

Objectives:
Identify those characteristics of surfaces and blood which make an atraumatic nonthrombogenic environment possible.

Major Findings:
Two intravascular test devices have been designed to permit in vivo testing of aritificial materials under controlled blood velocities. Particular parts of the device are isolated from contact with possibly damaged intima and consequent release of tissue thromboplastin. The role of various surfaces, turbulent flow, platelets, coagulation factors, temperature, and blood velocity on the initiation of clotting are being investigated. Various histologic chemical and physical manifestions of clotting are being measured. The effects of aspirin and methylene blue on clotting are also being studied.

Proposed Course:
Continue this multifacet bioengineering approach in an attempt to better define an artificial nonthrombogenic system.

Contractor: Cornell University Medical College

Amount: No funds in 1972

Title: Collagen Membranes and Surfaces: Their Application to Problems of Hemodialysis and Chronic Uremia

Objectives:

Investigate the properties of dialysis membranes and A-V shunts constructed from solubilized collagen.

Major Finding:

Tubular dialysis casing has been prepared and tested *in vitro* and *in vivo* with the conclusions that:

1. Collagen dialysis casing can be inexpensively prepared;
2. Current membranes have about the same permeabilities as cuprophane, but are two to three times as thick;
3. Blood compatibiilty, utilizing several different testing methods is poorer than cuprophane.
4. Animal studies have revealed no toxicity; and
5. Clinical dialyses reveal adequate but not outstanding performance as would be predicted from the *in vitro* data.

Collagen hollow fibers have been prepared from enzyme solubilized and purified collagen. Fibers can be made in various dimensions and bind well with silastic. Preliminary *in vitro* studies show them to have exceptionally high ultrafiltration rates, and good permeabilities to creatinine. In vivo testing with a heparinized, anesthetized dog revealed about 10% or less occlusion of fibers.

Proposed Course:

Since collagen offers no performance advantages this contract will terminate in 1972.

Contractor: The Dow Chemical Company

Amount: $56,500

Title: Research and Development of Cannulas and Nonthrombogenic Materials

Objectives:

Develop, fabricate, and evaluate a useful cannula for subcutaneous insertion between the arterial and venous ends of vessels. Continue investigation of dacron velour with appropriate diameter and thicknesses for inhibition of infections. Investigate coating of silastic cannulas with nonthrombogenic materials.

Major Findings:

1. It was found that the end to side anastomosis is apparently not feasible in small peripheral arteries with this investigating group.

2. Prototypes of the proposed cannulae are being fabricated and will undergo modification and in vivo testing in laboratory animals in the ensuing months.

This contract is due to terminate this year.

Contractor: Dow Chemical U.S.A. and University of California

Amount: $183,666

Title: Evaluation of Experimental High Flux Artificial Kidneys

Objectives:

Develop and clinically evaluate hollow fiber kidneys with improved clearance for higher molecular weight solutes.

Major Findings:

It is not known to what extent the morbidity characteristic of chronic dialysis therapy may be due in part to the low clearance of solutes, above molecular weight 200 in current artificial kidneys. Clinical study of this problem has been limited to date primarily because of the high ultrafiltration rate of membranes with suitable permeability to larger molecular weight solutes. Hollow fiber cellulose acetate membranes with greatly enhanced permability to water and large solutes have been developed and successfully used with a pressure balancing system to accurately control mean transmembrane pressure and ultrafiltration rate during clinical hemodialysis.

In vivo performance measurements on kidneys with markedly enhanced water and higher molecular weight solute permeability have been obtained. Ultrafiltration could be precisely controlled during clinical hemodialysis. Protein loss averaged .45 gm/hour in the $1M^2$ device and is anticipated to average .63 gm/hour in a $2M^2$ device. Solute clearance in the 1300 and 5000 MW range can be increased 400 and 900% respectively, with a controllable 4 to 40% increase in clearance in the 60 to 160 MW range. These kidneys provide a powerful tool to study the effect of markedly enhanced clearance of higher molecular weight solutes in chronic dialysis therapy. If important toxic compounds in this MW range are present in these patients therapy with these high flux kidneys could provide evidence indicating to what extent anemia, renal osteodystrophy, coagulopathies, protein malnutrion and peripheral neuropathy are potentially dialysis dependent abnormalities.

Proposed Course:

This important investigation of the clinical performance of high flux dialyzers will continue.

Contractor: Albert Einstein College of Medicine

Amount: $53,988

Title: The Structure and Function of the Hepatic Endoplasmic Reticulum in Chronic Renal Failure

Objectives:

Objective of this contract is to investigate the role that chronic uremia has in relation to the function of the hepatic endoplasmic reticulum. It is further hoped that through elucidation of the role of the endoplasmic reticulum an understanding may be made of the mechanisms of drug metabolism in chronic uremia.

Major Findings:

In azotemic animals prolonged drug metabolizing activity was demonstrated which probably reflects reduced drug hydroxylation capability. Detailed morphologic studies utilizing such tools as electronmicroscopy revealed consistent changes in hepatic ultrastructure in azotemic rats.

Proposed Course:

Continuation of current studies of hepatic endoplasmic reticulum function in control and azotemic animals.

Contractor: Envirogenics Company

Amount: $35,000

Title: Research and Development of Hemodialysis Membranes

Objectives: The contractor has been working with the objective of developing membranes for hemodialysis by using membranes of asymmetric cellulose or cellulose derivatives. He has also designed his work so that these membranes can be made cheaply and in large enough quantities for use with present hemodialysis equipment.

Major Findings: The contractor has successfully prepared asymmetric membranes of cellulose acetate, diethylaminoethyl cellulose acetate (DEAECA), and cellulose acetate hydrogen succinate (CAHS). These membranes have been extensively tested and cellulose acetate has been shown to have somewhat improved transport of sodium chloride, urea, and creatinine with superior transport of uric acid. DEAECA has facilitated transport of acidic compounds while CAHS has facilitated transport of basic compounds. Ultrafiltration rates remain significantly higher than presently available membranes and controllable. Patients have been dialyzed at the Wadsworth Veterans Administration Hospital with the use of the Aerojet asymmetric cellulose acetate membrane. The membrane proved to be the excellent ultrafilter predicted, caused no adverse reactions, and performed generally well. To date 500 clinical dialyses have been completed using the cellulose acetate membranes and good results have been achieved.

Proposed Course: To produce sufficient quantities of cellulose acetate membranes so they can be distributed to dialysis centers in the U.S. for clinical trials.

Contractor: Envirogenics Company

Amount: ------

Title: Development of an Inexpensive Manufacturing Method for the Kiil-Type Artificial Kidney

Objectives: To develop a low-cost manufacturing process for the Kiil-type artificial kidney.

Major Findings: The low cost production method consisted of compression molding the grooved surfaces of the boards and bonding these thin plastic parts to a lightweight stiffening member. The combination constitutes a complete Kiil-type board having critical dimensions identical to those of commercially available milled boards.

Compression molding, using either polyvinyl chloride or polypropylene, was selected to produce the grooved face plates of the boards. This technique assures flat moldings free of distortion and stresses. It also assures perfect reproduction of the minute, sharp-cornered details of the grooves.

The development of the molds, using electroforming techniques, was a major program task. The method selected offers the advantages of ease of replication, matchline-free moldings, and excellent reproduction of fine, intricate geometrical patterns. The development task started with fabrication of a master from a 1-inch-thick plate of stainless steel. The grooves were machined into the face of the plate master by using the crush form grinding process. The pattern was established by a statistical analysis of the dimensions obtained from a set of commercial Kiil boards. A mold was made, duplicating the grooved section of the master, by electroforming. Using this mold, with a 600 ton press, face plates made of polyvinyl chloride were produced. Samples were also molded from Implex-F and polypropylene. The stiffiner, to which the compression molded face plates were adhesively bonded, was a glass reinforced polycarbonate injection molding. It was a hollow grid configuration with the symmetry required to prevent distortion.

Absence of water leachable toxins from the materials employed was verified by animal tests. Swiss white mice were injected intraperitoneally with sterile saline extracts obtained from all the materials employed.

The mold production by the electroform process appears to be an excellent avenue to arrive at precise reproduction of support geometries and may be the only solution to ultra-fine support geometries that, while machineable in the positive, are impractical to make the negative which the mold requires. The composite master produced by a combination of machining operations which may or may not include crush from grinding is an excellent starting point.

Compression molding from thermoplastic powders has proven to be a good process choice. It will show further advantages in point support geometries where air entrapment at the apex of each individual cone or pyramid might tax other molding processes beyond their capabilities. The use of higher softening point polymers which have become more available, such as polypropylene in higher molecular weights, will contribute to simplifying the assembly problems by broadening the choice of adhesives and permitting the use of bond films. The latter can be an important factor in economic production of the boards.

On the basis of the manufacturing plan developed in the course of the program, the production costs per set of plates of less than $250 can be forecast. These costs compare very favorably with the current list price of $1,000 for commercially available full size Kiil boards.

Proposed Course: Since the basic objective of the contract was completed and the method can be partially or totally adapted to new generations of flat plate dialyzers, the contract was terminated.

Contractor: Georgetown University

Amount: $65,400

Title: New Approaches to Design of Chronic Dialysis Cannulae for the Prevention of Infection

Objectives:

1. Develop a method of fixation of an AV cannula to minimized turbulence related thrombosis in cannula dislodgement due to vessel nephrosis.

2. Develop an antithrombogenic material for use in the AV cannula.

3. Prevent cannula sinus tract formation by means of developing a percutaneous seal for the skin prosthetic junction.

Major Findings:

Arterio-venous shunts coated with hepacone have proven to be thrombo resistant in dogs and a unique vascular shunt anastomosis method has reduced junctional clotting in animal experiments. A novel percutaneous button seal has been developed and tested in animals and appears to be a promising approach to prevent infection of the sinus tract formation. The shunt survival with this group seems to be as great as with any group investigating cannula survival at the present time.

Proposed Course:

Continue development of a tissue compatible nonthrombogenic AV shunt. Continued clinical trials will be carried out.

Contractor: Gulf South Research Institute

Amount: $97,300

Title: A Study of Synthetic Polypeptides as Possible Hemodialysis Membranes

Objectives: To produce a polypeptide (poly-γ-methyl-D-glutamate) membrane for an in vivo clinical evaluation study.

Major Findings: Previous work in this project showed that membranes for hemodialysis use could be prepared from a number of poly-α-amino acids and that their transport properties are a function of their composition. The investigation has elucidated the relation between the various membrane properties and their composition. During the current contract period emphasis has narrowed to application of neutral poly-α-amino acids for clinical utilization. One polymer has been selected for clinical trials. Because of the advantages in physical properties, the cost and availability of the polymer, the poly-γ-methyl-D-glutamate film was chosen as the first type membrane to produce for clinical trials. To improve the reproducibility of the membrane properties, they are currently working with a commercial firm to develop a continuous casting procedure. This membrane is prepared in a dry form; it has satisfactory blood compatibility; it has excellent mechanical properties; and it has a promising pattern of permeability, especially for higher molecular weight solutes. Its permeability can be altered to provide a group of membranes with diffusing ranges of permeabilities.

In preparation for extended clinical trials, they have established blood compatibility tests; conducted animal dialysis trials; and conducted initial dialysis trials in humans. Patient studies were conducted uneventfully with one methyl-D-glutamate membrane, first in a Babb-Grimsrud Cell in a satellite circuit during on-going Kiil hemodialysis, and then in mini-Kiil dialyzers with cuprophane in one layer and the polypeptide membrane in the other layer. This membrane had passed all animal trials and dialyzer assembly tests. Further patient trials are in progress.

A group of chronic hemodialysis patients are being maintained in a stable clinical state which is well characterized by many test parameters in readiness for study of the effects from long-term use in hemodialysis of this group of membranes.

Blood compatibility studies centered on four aspects concerning thrombogenicity: (1) further standardization points were made on the multiple clotting cell method of Lindholm (MCCML) which is used to compare thrombogenicity among various membranes, documenting the direct linear relationship of clotting time to the blood volume/surface area ratio in the cells, the consistent relative thrombogenicity of the standard reference

surfaces (glass versus siliconized glass and cuprophane) employed in each test series, the relative magnitude of clotting times in the test cell versus standard Lee-White clotting tubes (2:1), and the linear scales of clotting time prolongation resulting both in the Lee-White glass tubes and in Lindholm test cells (glass and cuprophane) from the presence of known amounts of aqueous sodium heparin. The latter has application to studies of elution rate of heparin from heparinized surfaces; (2) differential thrombogenicity relative to loss, siliconized glass, and cuprophane has been defined by MCCML for over 85 experimental membranes, heparin treated and untreated, as screening preliminary to in vivo testing; (3) heparin treated membranes have been under study by this method (MCCML), reliably showing success or failure of the heparinization processes being tried and demonstrating the rate of heparin elution upon blood exposure. All studies to date have shown that the non-thrombogenic effects of heparinization of membranes is directly related and solely due to the degree of heparin elution; (4) this method (MCCML) is also being used to compare clotting propensities among all dialysis patients, with special reference to Cuprophane PT150 and experimental membranes as compared to glass surface, hoping to find clues to hypercoagulable states in patients and any patient-specific clotting tendency among the various membranes.

Proposed Course: The objectives for the proposed research period are:

1. To develop the process conditions which will result in high yield usable membrane production of polypeptide membranes on casting equipment made available through Helena Laboratories, Beaumont, Texas.

2. To use G.S.R.I. laboratory continuous casting facilities to identify composition abd casting environment parameters which will allow the maximum choice in the resulting membrane properties.

3. To clinically evaluate these membranes in terms of the long-term effects on a chronic hemodialysis patient.

INTERNATIONAL RICE RESEARCH INSTITUTE (PH-43-67-726)...U.S.-Japan Program

Amount: $54,060

Title: Improvement of the Protein Content of Rice

Project Director: Dr. B. Juliano

Project Officer: Dr. B. T. Burton (NIAMD)

Objectives:
The primary objective of this project is to determine the feasibility of incorporating a high level protein into commercially produced rice in Southeast Asia. Presently used commercial varieties of rice contain from seven to seven and one-half percent protein. The biological quality of rice protein is fairly high and an increase in the protein level in rice would go far toward alleviating protein undernutrition in countries for whom rice is the mainstay of subsistence. Since 60 percent of the world's population subsists primarily on rice, the implications for an amelioration of the chronic protein shortage in many underdeveloped countries, should this project prove successful, are quite obvious. If the present protein level of rice could be elevated by several percentage points an automatic and sweeping nutritional improvement would take place in major areas throughout the world.

Methods Employed:
The modus operandi involves the screening for high protein content, of over 7,000 varieties of rice archived in the International Rice Research Institute collection, followed by selective breeding and development of a commercially feasible variety which incorporates an improved protein level together with all the necessary attributes which would make this rice attractive to the average Southeast Asian rice grower, namely high yield per acre, resistance to endemic diseases, strong response to fertilization, and--most important--desirable organoleptic qualities with respect to stickiness, mouth-feel and taste.

Major Findings:
Over 100 rice varieties have been identified among the many thousands tested which contain between 13-1/2 to 17-1/2 percent protein. Thus far six high protein strains of rice (some from Hungary and some from Southeast Asia) have been successfully crossbred with a new rice variety (IR8) which has been developed previously at the International Rice Research Institute and which possesses near-ideal plant characteristics including disease resistance and a very high yield per acre. Some of the new hybrid lines have shown as high a protein content as the high-protein parent. Others, of fairly acceptable "plant type" have a protein content of +-11 percent, i.e., contain 50 percent more protein than currently produced commercial rice. Based on the most recent breeding work involving progeny of successful crosses between the six high-protein strains and IR8 the project staff is encouraged that the protein content of rice is indeed a genetically determined characteristic.

Protein efficiency ratio (PER) studies, using rats, of several parent rice strains have been carried out by Dr. Bressani at the Institute of Nutrition of Central America and Panama (INCAP) and future PER studies of the new hybrid varieties are contemplated. Dr. Helen Clark of Purdue University has just finished a series of human nitrogen balance studies utilizing one of the experimental high protein rice strains as the source of dietary nitrogen. This strain (BPI 76-1) was compared in adult subjects with an equal weight of rice of the commercial "Bluebonnet" variety at a daily intake of 480 grams. The experimental rice which contained 1.8 times as much protein as Bluebonnet rice, caused significantly higher nitrogen retention ($P < 0.01$), which is attributable to the increased amounts of essential amino acids. The respective mean positive balances were 1.41 = 0.89g and 0.24 = 0.31g. Addition of supplementary nitrogen to make the Bluebonnet rice isonitrogenous with the BPI-76-1 variety did not cause a statistically significant improvement. All subjects were in negative balance when 320g Bluebonnet rice were made isonitrogenous with 480g of the same rice by adding nonspecific nitrogen.

Significance:

(Please see above under Objectives.) Ordinary commercial rice does not contain enough protein to support normal growth and development of infants and children maintained on a predominant rice diet, and does not protect the adult population from serious protein under-nutrition. Rice is the principal food of 60 percent of mankind. If the present protein level of rice (approximately 7-1/2 percent) could be elevated by several percentage points an automatic and sweeping nutritional improvement would take place in all the populations for whom this one crop represents the nutritional mainstay. Such a development would concomitantly relieve the current burden of the United States which supplies hundreds of millions of dollars' worth of supplementary foods to underdeveloped rice-eating countries. The potential nutritional improvement among Southeast Asian populations and underdeveloped rice-eating countries throughout the world is an important aim of the U.S.-Japan Program and of the applied nutrition program of the Institute.

Proposed Course:

The high protein rice lines isolated thus far have about a 5 percent lower field yield than the IR8 "wonder rice" and are somewhat more susceptible to leaf blight. Therefore, cross-breeding experiments will continue to select out high protein strains with improved field qualities. In the face of the encouraging progress thus far, further crossbreeding and growing of the resulting hybrids in large plots is contemplated, side-by-side with additional protein efficiency ratio determinations, in rats, and human nitrogen balance and digestibility studies, of the protein of the resulting hybrid crops.

Contractor: The Johns Hopkins University

Amount: $46,000

Title: Metabolic Studies in Uremia

Objectives:
To evaluate the effect of dialysis upon the rate of cortisol secretion in chronic uremia; and to investigate the possible role of growth hormone in the carbohydrate intolerance of uremia; and any effect which dialysis might have upon these abnormalities.

Major Findings:
1. Observations of the pattern of cortisol secretion (in plasma) at 15 minute intervals during the first four hours of hemodialysis showed that there were episodic bursts of cortisol secretion which ranged in duration from 15 to 45 minutes. Simultaneous measurement of dialysate cortisol levels did not show the rapid changes in its levels that were noted in the plasma.

2. Intravenous glucose tolerance tests (GTT) with concomitant measurements of growth hormone levels were made immediately prior to the initiation of maintenance hemodialysis and after stabilization on hemodialysis. Pre-dialysis growth hormone levels during the GTT, the following were noted: control-14.5 mμg/ml plasma; 30 min-19.4 mμg/ml; 60 min-24.5 mμg/ml while in the patients stablized on dialysis, the hormone level decreased to 9.8 mμg/ml plasma without a tendency to rise during the GTT. It is postulated that the abnormal GTT may be related in part to the elevated levels of growth hormone.

Proposed Course:
1. Cortisol levels are being studied to correlate them in the same patient on and off dialysis.

2. Techniques are being developed for measurement of cortisol binding to serum proteins.

3. An assessment is being made of the usefulness of monitoring growth hormone as an indicator of adequacy of dialysis.

Contractor: Arthur D. Little, Incorporated

Amount: $38,200

Title: Elucidation of Toxic Nature of Uremia by Application of the Spin Filter Culture System

Objectives:

To elucidate the toxic nature of uremia by application of the Spin Filter Culture System. By means of this system, mammalian cells can be propagated in suspension and the uremic toxins present in dialysate may be chronically administered to these cells.

Major Finding:

Normal dialysate and dialysate with an increased concentration of urea and creatine were found to have no adverse effect on the Spin Filter Culture System.

Proposed Course:

To test dialysate from uremic patients in the Spin Filter Culture System.

Contractor: Massachusetts Institute of Technology

Amount: $87,900

Title: Non-Thrombogenic Surfaces and Artificial Kidney Design

Objectives:

To develop non-thrombogenic surfaces for a pilot model of an artificial kidney.

Major Findings:

Studies of nonthrombogenicity of formaldehyde-glutaraldehyde-hydrogels covalently bonded with heparin indicate that they are materials of low thrombogenicity. In vivo studies, including A-V shunts on dogs, and rings in the inferior vena cava according to the Gott model, both fixed and floating, indicate that covalently heparinized surfaces will not be practical for the large areas of artificial kidney devices unless these surfaces are previously coated with serum albumin, to avoid platelet attraction.

In vitro tests by themselves are necessary but insufficient for predicting ultimate in vivo performance. (The covalently heparinized hydrogels that we have tested, even without prior exposure to albumin, exhibit excellent performance in in vitro tests: all blood clotting times exceed 50 minutes, no elutable heparin is found, insignificant plasma proteins are adsorbed, and blood clots normally when transferred from test tubes to standard activating surfaces.)

Hydrogel membranes have been greatly improved by casting the reacting solutions on the non-woven nylon fabric Cerex (TM: Chemstrand). 5.3 mil hydrogel membranes show equal resistance to sucrose (100 min/cm) as 1.1 Cuprophane and twice the resistance to urea (32 vs. 17 min/cm). Potentially useful tubing and other hardware have been prepared by modifications of the original casting syrup.

Proposed Course:

Although surface properties of heparinized hydrogels are promising, the mechanical properties are generally poor. This contract will terminate in 1972.

Contractor: Mayo Foundation

Amount: $30,524

Title: Metabolism of Creatinine and Guanidine Compounds

Objectives:

1) To determine the extent to which creatinine is catabolized in normal and in uremic man, and the physiologic significance of creatinine metabolites.

2) To determine if there is a positive correlation between serum and urine guanidine concentrations and uremic symptoms in uremic patients.

3) To determine if the increased serum guanidine concentration in uremia is related to abnormal nitrogen metabolism; and also the metabolic source of the guanidines in body fluids of the uremic patient. If there is correlation between serum concentrations of guanidine and the uremic symptomatology, an attempt will be made to identify "points of control" in the metabolic sequence leading to guanidine formation in this group of patients.

Proposed Course:

The contribution of the microflora of the gastrointestinal tract to the development of the uremic syndrome and the relationship of dietary methods of controlling uremic symptomatology to the microflora will be studied. Quantitative measurements of guanidines in body fluids of patients with diminished renal function will be studied in the above situations; as well as attempts made to induce "creatinase" activity in the microflora of the rat GI tract.

Contractor: Mayo Foundation

Amount: $91,585

Title: Value of Maintaining Parathyroid Hormone Suppressive Calcemia in Prevention of Bone Disease and Abnormalities in Calcium Hemeostasis in Patients on Long-Term Hemodialysis

Objectives: This project is directed toward assessing the value of maintaining physiologic plasma concentrations of both calcium and phosphate in suppressing parathyroid hormone secretion and preventing metabolic bone disease and abnormalities of calcium homeostasis in patients maintained on long-term hemodialysis. Parathormone assays in relationship to varying levels of dialysate calcium, dietary calcium, and possible vitamin D supplementation will be studied. Parathormone assays in relationship to phosphate which had been lowered by oral administration of aluminum carbonate and hydroxide will also be studied. Bone biopsies and bond density studies will be performed.

Major Findings: Thus far, the dialysate Ca level has been identified as a critical factor in the pathogenesis of renal osteodystrophy. In addition,it has been found that maintenance of serum phosphate near normal levels with phosphate-binding gels is crucial. Serum PTH levels respond to changes in these critical factors.

Proposed Course: Evaluation of dialysate Ca concentration of 8 mg %, its effect on PTH, and follow-up of patients receiving renal transplantation.

Contractor: Mayo Foundation

Amount: $101,140

Title: "Uremic Polyneuropathy: (a) Instrumentation and Quantitation of Sensation as an Index of the Effectiveness of Dialysis and, (b) Histologic and Lipid Evaluation of Biopsied Nerve".

Objective: The new contractor will:

(1) Build instruments for the quantitative measurement of touch-pressure and warm/cold sensations.

(2) Test the usefulness of these new instruments in detecting the presence of mild uremic neuropathy by comparing them to currently available methods.

(3) Use these quantitative measurements as an index of the severity of neuropathy in patients treated by a standard dialysis regimen and those on infrequent dialysis.

(4) Use histologic and biochemical studies of nerve and liver tissue to elucidate pathogenic mechanisms in the development of uremic neuropathy.

Contractor: Minneapolis Medical Research Foundation

Amount: $56,200

Title: Investigations of Nutritional Requirements of Chronic Renal Failure

Objectives:

This is a contract designed to delineate nutritional requirements of patients with chronic renal failure treated conservatively and by long-term hemodialysis including dietary protein requirement to maintain optimal body composition and body weight as well as specific amino acid requirements.

This contract is due to terminate this year.

Contractor: University of Minnesota

Amount: $44,998

Title: Fluid Dynamics of Blood Cells

Objectives:

This is a new contract with five objectives:

1. Analysis of blood velocity profiles in narrow channels using a 3-D microscope system.

2. Study of hematocrit skimming layers using fluid conductivity measurements.

3. Couette skimming layer force evaluation.

4. Analysis of flow enhanced diffusion coefficients using the Taylor Dispersion Method.

5. Studies of formed element behavior following wall contact using electron and phase microscopy.

Contractor: Montreal General Hospital

Amount: $63,019

Title: Determination of Nutritional Requirements and the Study of Body Composition in Patients on Chronic Hemodialysis.

Objectives: Dr. Kay's objectives are to determine the nutritional requirements and to study the body composition of patients on chronic hemodialysis.

Major Findings: The investigator has demonstrated that renal osteodystrophy may respond well to dihydrotachysterol (DHT) and he has successfully reversed progressive bone disease in dialyzed patients studied thus far. He has also studied fluoride levels in patients undergoing dialysis in both fluoridated and non-fluoridated areas and has found no difference in the degree and type of bone disease in these two populations.

In addition he has undertaken preliminary animal studies using the uremic rat.

Proposed Course: Extensive animal model studies to systematically determine the causative factors of renal osteodystrophy in well-controlled studies.

Contractor: University of Naples

Amount: $16,000

Title: Studies on Amino Acids in Uremia

Objectives:
To study selected facets of amino acid metabolism in chronic uremia with emphasis on phenylalanine, tryosine and histidine. To establish the true essential amino acid requirements in chronic uremia [which differ from those of normal man]. To investigate the feasibility of special diets for uremic patients based on keto acid analogues of the amino acids essential for maintenance in uremia.

Major Findings:
The principal investigator has demonstrated that in chronic uremia the ordinarily nonessential amino acid histidine is indeed essential. Histidine supplementation improves uremic anemia and nitrogen balance. Based on data obtained to date, glycine appears also to be an indispensable amino acid for hemoglobin synthesis in uremia. Preliminary feasibility studies have indicated that uremic patients can utilize the keto acid analogues of essential amino acids in endogenous protein synthesis.

Proposed Course:
With the aid of dietary and metabolic studies and other relevant experimental investigations in uremic patients the contractor will establish the true essential amino acid requirements in chronic uremia [which differ from those of normal man]. Subsequently, the contractor will investigate the potential usefulness of special diets based on all the amino acids found to be "indispensable-in-uremia" for improvement of nitrogen balance and hemoglobin status of uremic patients.

The contractor will also investigate the possible utilization by uremic subjects of keto acid analogues of essential amino acids in endogenous protein synthesis. Lastly, the contractor will investigate problems of nitrogen storage under normal conditions and in uremia by tracing free and conjugated amino acids in various tissue compartments.

Contractor: University of Naples

Amount: $20,340

Title: Studies on Oxystarch in the Treatment of Uremia

Objectives:
To develop a chemical compound which can be administered per os to sequester urea and possibly other uremic toxicants in the gastrointestinal tract, and to develop with the aid of such a compound a suitable adjunctive treatment for patients in chronic renal failure. Also, to explore the feasibility of using oxystarch as a sorbent of uremic wastes in artificial kidney dialysate solutions.

Major Findings:
A chemically well defined polyaldehyde has been obtained from controlled periodate oxidation of starch. The material does not contain iodine residues and has been proven nontoxic in long-term mouse feeding experiments. The material is stable and non-dialyzable. One hundred grams of "oxystarch" bind 33 grams of urea. When given by mouth to uremic patients in divided doses, 20 grams of oxystarch per day result in a daily reduction of 20 milligram percent of blood urea nitrogen. Fecal nitrogen content increases correspondingly and the increased fecal nitrogen can be ascribed to large quantities of urea irreversibly trapped by oxystarch during passage of the gut. Oxystarch is not hydrolyzed in the intestinal tract, nor absorbed. Twenty grams of oxystarch when given daily in three to four divided doses to uremic patients maintained on the Giordano-Giovannetti diet caused a progressive daily lowering of blood urea nitrogen of 13 milligram percent (range 8 to 22); the corresponding increase in nitrogen content of the feces had a net mean value of 1,450 milligrams per day (range 730 to 8,050). When oxystarch was given to a patient on regular 3-times-weekly hemodialysis, the interdialytic interval could be prolonged up to 7 days without untoward effects. When oxystarch was given to a patient maintained with weekly peritoneal dialysis it was possible to space these treatments 25 days apart.

Dr. Giordano's laboratory data on urea binding with oxystarch at pH 1 to 1.2 and pH 7.4 have been corroborated by Dr. Robert Sparks at Case Western University in in vitro experiments.

Proposed Course:
The investigator will test the use of OXS in controlled larger clinical trials for prolonged periods of time in accordance with carefully outlined plans. Also, the contractor will prepare oxystarch in quantities large enough for clinical and laboratory experimentation in the United States as specified by the Artificial Kidney - Chronic Uremia Program which will distribute this material to American investigators for specific, sponsored studies.

Contractor: National Institute for Scientific Research

Amount: $117,200

Title: Development of Modified Polycarbonate Membranes for Hemodialysis

Objectives: To develop polycarbonate hemodialysis membranes with mechanical and transport properties superior to those of Cuprophane.

Major Findings: The bis-phenol A/polyethyleneglycol 6000 polycarbonate containing 25 wt% of the polyether component was chosen for initial dialysis membrane fabrication on the basis of water sorption and mechanical properties of representative formulations. Gelled membranes were prepared from this copolymer via the phase inversion technique using chloroform as casting solvent and methanol as gelation medium. Dimethyl sulfoxide (DMSO) was investigated as a swelling agent in the casting formulation. Ultrafiltration and dialysis measurements were conducted with these membranes and Cuprophane controls in an NBS dialysis cell, employing a series of 10 solutes of increasing molecular weight. Data were obtained at 25.0 $\pm$ 0.1°C. and zero pressure differential, using isotonic saline as the medium for the original dialysate and feed solutions. Ultrafiltration rates were measured at 600mm transmembrane pressure with 1000 ppm albumin (Bovine Fraction V) solution, as well as deionized water, to determine the degree to which the membranes rejected the protein. No albumin could be detected in the filtrates. These data indicated marked superiority over Cuprophane of the polycarbonate membranes cast from the formulations containing DMSO (2 and 4 gms DMSO/15 gms polymer in casting solution), particularly in the transport of the higher molecular weight solutes, no albumin leakage, and with ultrafiltration rates maintained at a level 2 to 5 times that of Cuprophane. The efficacy of DMSO as a swelling agent was clearly established.

Blood compatibility tests of one of the polycarbonate membranes indicated its thrombogenicity to be comparable to that of Cuprophane and siliconized glass, on the basis of whole blood clotting times. Toxicity screening of polycarbonate membranes by three different test procedures revealed no toxic effects.

Proposed Course: Future work will emphasize scaled-up copolymer synthesis and preparation of larger quantities of polycarbonate membrane for clinical testing. Supporting studies are continuing to develop methods for drying and rewetting of membranes, and to determine the effects of copolymer composition and molecular weight, and formulation and fabrication variables on membrane properties.

Contract: New York Medical College

Amount: $160,561

Title: Toxins in Uremia

Objectives:

The original objective of this project was evaluation of a special protein restricted diet for the alleviation of the subjective and objective signs and symptoms of uremia and an attempt to use this diet to elucidate the toxic factors in uremia. This objective has been expanded to include a search for the toxic factor(s) responsible for the transketolase inhibitor in uremic serum.

Major Findings:

In the first four years of this contract the New York Medical College group have treated 70 patients with a modified Giovannetti-Giordano diet. In patients with GFRs of greater than 4ml/min, dietary therapy was usually adequate in reducing gastrointestinal symptoms and increasing the patients' comfort although it in no way slowed the progress of the underlying renal disease or its complications. Studies were also undertaken to prolong the interval between dialyses in patients with GFRs of less than 4ml/min. Diet-dialysis therapy was always associated with the eventual development or worsening of motor neuropathy which required intensive dialysis for reversal. Pre- and post-dialysis serum amino acid levels have been determined in 17 patients.

Because neurological signs and symptoms play such an important role in the uremic syndrome, and because some of the neurological manifestations in the patients resembled those noted in other conditions, the investigators recently began a search for identifiable metabolic correlates which might in some way explain the neuropathy. The investigators discovered that transketolase activity was found to be reduced by an inhibitory factor acting directly on transketolase which is found in the blood and dialysates of uremic patients. Transketolase was determined in 15 chronic uremic patients before and after four to six hours of hemodialysis and in four patients with advanced renal disease before and after institution of G-G diet therapy. In all except two patients on hemodialysis there was a significant increase in transketolase activity after dialysis. In patients on the G-G diet, transketolase values were initially low and remained low even after considerable time on the diet (36 weeks in the case of one patient). The responsible factor(s) was concentrated and separated into various molecular weight fractions. Only fraction I (mol wt less than 500) produced significant inhibition.

The results of these investigators indicate that a substance or substances inhibiting the activity of the enzyme transketolase, for

which thiamine pyrophosphate is the coenzyme, is found in the plasma of chronic uremia patients exhibiting signs of peripheral neuropathy. This inhibitor is not significantly diminished by diet therapy, but can be removed by hemodialysis. The enzyme affected occupies a central position in the pentose phosphate pathway of non-oxidative glycolysis. The demonstration of an inhibitor of erythrocyte and brain transketolase in plasma from uremic patients suggests a possible mechanism for the development of uremic neuropathy, and its response to hemodialysis. The contractor feels that this enzyme model system may offer a technique for investigating the metabolic lesions of uremia and may serve as an objective measure of response to dialytic therapy.

Proposed Course:

The investigator will continue studies to identify the toxic materials and extend the clinical and biochemical evaluation of the transketolase inhibition.

Contractor: University of North Carolina

Amount: $51,500

Title: Antithrombogenic Surfaces: Platelet-Interface Reactions

Objectives:
Study the composition of the layer of plasmatic components adsorbed to materials exposed to blood, and to characterize this layer as to its effect on blood platelets.

Major Findings:
These investigators indicate that there are morphological differences between the adsorbed layers formed on certain polymeric and nonpolymeric test materials and that an adsorbed layer is universally present between adherent platelets and the surface of the test material. It appears that it is this adsorbed layer and not the surface of the test materials which determines thrombogenicity. The chemical nature of the test material may control the composition of the adsorbed layer and through this, its thrombogenicity, or it may merely control the rate of deposition of the adsorbed layer.

Proposed Course:
Continue characterization of the blood-polymer layer and its role in material thrombogenicity. He is also going to evaluate various polymers in an *in vivo* situation by placing vena cavae rings in Rhesus monkeys.

Contractor: North Star Research and Development Institute

Amount: $105,000

Title: Development of a New Concept in Membrane Structure for Application in Hemodialysis

Objectives:

The objective of the contractor has been to develop ultrathin (5,000-20,000 angstroms in thickness) membranes for hemodialysis.

Major Findings:

The most promising membrane discovered thus far is ultrathin cellulose. Ultrathin cellulose (when supported) had approximately twice the dialysis rates of cuprophane and about three times the ultrafiltration rate of cuprophane. The membrane did not pass albumin and had approximately equal dialysis permeabilities for bacitracin. In addition, the contractor has developed techniques that enable large amounts of the ultrathin polymer to be manufactured at minimal cost. This would enable the membrane to be useful commercially.

Proposed Course:

Completion of *in vivo* animal testing of the membrane and support composite in preparation for clinical testing. Also, to initiate clinical testing.

FOOD COMPOSITION TABLE FOR USE IN EAST ASIA . . . . U.S. - Japan Program

Amount:
Interagency Agreement with the Nutrition Program, HSMHA: $10,600

Contract for additional personnel with the American Institute of Nutrition (NIH 70-2086): $39,118

Project Director: Dr. W. T. Wu Leung, Nutrition Program, HSMHA

Project Officer: Dr. B. T. Burton, NIAMD

Objectives:
The primary objective of this project is the preparation of a table of food compositions for foods used in the East Asian countries (Burma, Thailand, Laos, Vietnam, Cambodia, Malaysia, Indonesia, Philippines, Taiwan, Korea, and Japan). This includes indigenous food products as well as foodstuffs imported to these countries which are consumed locally. The first Far Eastern Food Table was issued in 1945 under the auspices of the Committee on International Food Value Problems of the National Research Council, U.S.A. Although it was revised and enlarged in 1952 as Agriculture Handbook No. 35, "Composition of Foods Used in Far Eastern Countries", it is not applicable to conditions in 1969. Comprehensive food tables for local foods in East Asia are essential for and basic to any attempt of improving the nutritional state of East Asian populations through a combination of nutrition education or nutrition improvement through introduction of innovations in the local food consumption.

Major Findings:
The Principal Investigator, Dr. Leung, who was responsible for the preparation of the first Far Eastern Food Table immediately after World War II, is collecting all available food data through correspondence and through personal visits in the various countries. In addition, there is active collaboration with the Nutrition Division of the Food and Agriculture Organization of the United Nations, through Dr. Autret and his staff who have accumulated a considerable body of data at FAO Headquarters in Rome and are constantly generating new information in regional FAO offices in Asia.

The information collected includes: data on moisture, "proximate composition", minerals, vitamins, amino acids, fatty acids, trace elements, refuse, and local methods of preparation, processing and preservation of foods, etc. Whenever feasible, local names used in various Far Eastern countries with the corresponding English and scientific names are recorded and collected. The result of this Project will be printed as a joint publication "Food Composition Table for Use in East Asia" - NIAMD will be responsible for the printing of the English version, and FAO will undertake the translation and printing in French. The format of the food table will follow closely the one previously designed for Africa.

Data collection was begun in the winter of 1969-70 and has progressed very satisfactorily. These have been evaluated and collated into tentative food tables with a view toward "freezing" of the tables and format in the fall of 1972.

Significance to NIAMD Program and Biomedical Research:
Please see "Objectives".

Proposed Course of Project:
It is expected that data collection will proceed at a reduced rate and will cease by late summer of 1972. Thereafter tables will be assembled and coordinated. A final editing will be given to this material and the format during a joint meeting with the FAO counterparts in the late fall of 1972, and a printer-ready copy produced thereafter.

Contractor: University of Pennsylvania

Amount: $189,541

Title: Blood Purification by Ultrafiltration and Reconstitution

Objectives: To study the factors involved and develop a system for cleansing the blood by ultrafiltration and replacing the ultrafiltrate with sterile reconstituting solution containing appropriate concentrations of normal body constituents removed in the ultrafiltrate.

Major Findings: Work has been largely completed on the theoretical formulation of concentration polarization of ultrafiltered protein containing solutions. This phenomenon was previously identified as the single most important limitation encountered during the first year's study of the feasibility of diafiltration as blood cleansing technique. The concentration polarization model formulated has been tested by varying such parameters as temperature, shear rate, protein concentration and pressure. Ultrafiltration rate was found to rise with increases in working temperature and blood path shear rate, to fall with increasing protein concentration and remain unchanged when transmembrane pressure gradient is varied above a plateau figure of 15 p.s.i. This performance substantiated the validity of the concentration polarization model.

A hollow-fiber configuration has been utilized an animal studies which have demonstrated the feasibility of acute hemodiafiltration. Clearance of large molecules (MW 5000) was nearly 90%, and that of smaller molecules nearly 100%.

A reconstitution system and a flow-pressure monitoring system has been developed that is operationally successful.

Proposed Course: Additional animal studies will be undertaken to prevent toxicity. A clinically practical reconstitution system will be explored, and clinical testing will evaluate the efficacy of the process.

Contractor: University of Pisa

Amount: $25,185

Title: Effect of Dietary Treatment on Albumin and Urea Metabolism in Chronic Uremia

Objectives:
To determine the effect of the dietary treatment on albumin and urea metabolism.

Major Findings:
Sophisticated determination of albumin catabolism through a new triple tracer technique has shown that the intravascular albumin mass is normal in patients on the low, selected protein (Giordano-Giovannetti) diet (even in patients maintained on it up to five years). The extracellular albumin mass, total albumin mass and extracellular to intravascular ratio were reduced in both short-term and long-term patients. The total intravascular albumin mass was preserved within a normal or near normal range. Albumin metabolism was normal in controls and long- or short-term patients. The diet is not directly responsible for the observed albumin depletion since albumin synthesis continues at a normal rate in all cases. Apparently toxic factors of the uremic state itself are responsible.

Proposed Course:
Continue to evaluate albumin metabolism in patients who are uremic and on a low protein diet, investigate the urea metabolism in these patients, and study albumin and insulin turnover and distribution in uremic patients who are on hemodialytic therapy.

Contractor: University of Pisa

Amount: $22,036

Title: Role of Guanidines and Related Compounds in Uremic Syndrome

Objectives:
To investigate the role of guanidines and related compounds in the uremic syndrome, specifically in relation to their retention in tissues, their abnormal metabolism, pathogenic effect on the physiology of different organ systems, metabolism and homeostasis, genesis and removal by dialysis.

Major Findings:
The principal investigator has found that methylguanidine (MG) accumulates in dogs and man in chronic uremia and causes in dogs a condition closely resembling advanced chronic uremia in man. MG depresses calcium absorption, raises plasma triglycerides and decreases plasma cholesterol, impairs the immunologic potential but does not affect glucose metabolism. It appears to be derived from creatinine (which is elevated in uremia) as well as from dietary muscle proteins, but not from eggs, milk and organ meats.

Proposed Course:
The contractor will identify and quantitate the various types and amounts of guanidines and related compounds in various tissue compartments of uremic patients and experimental animals.

He will also study the normal and abnormal metabolism of guanidinium and related compounds as affected by uremia and will study their interrelationships and interconversions and degradations.

The toxicity of these compounds for various parameters of metabolism will be investigated and their retention in uremia and removal by various modes of dialysis will be studied.

Contractor: Research Triangle Institute

Amount: $86,985

Title: National Dialysis Registry

Objectives:
To develop a national registry of all patients on hemodialysis.

Major Findings:
The National Dialysis Registry collects data from virtually all centers engaged in dialysis in the United States, and prepares from the data statistical analyses for use by the NIAMD and quarterly reports to all cooperating centers. The data include numbers of patients in center or home dialysis, changes in status of these patients, cause of death in patients that expire, length of time on dialysis, etc. Confidentiality of the center and patient identities is maintained.

During the past year analyses of death by primary cause and multiple decrement life tables have also become available.

The establishment of the Registry, although primarily devoted to vital-statistics fact finding, will allow, should more in-depth be required, the broadest possible base for statistically valid sampling which would not put a large load of data collection on any one center.

Contact is also maintained with the European Transplant and Dialysis Registry, and efforts are being made to make the data on dialysis compatible so that direct comparisons will be possible.

Proposed Course:
To continue obtaining, cataloging, storing, and making available data of patients on dialysis.

Contractor: Research Triangle Institute

Amount: $95,200

Title: Improved Non-thrombogenic Materials

Objectives:

Goals are to prepare a number of potential non-thrombogenic surfaces by grafting hydrophilic polymer chains to other polymeric substrates, and to test such surfaces for blood compatibility in vitro. The investigators will prepare a series of hydrogels of varying water content and test these by standrard in vitro coagulation studies.

Major Findings:

The substrate material used for grafting has been polyethylene in the form of film or tubing. The grafting of hydrophilic monomers to polyethylene has been accomplished by two procedures. One involves irriadiation of substrate in an aqueous solution of monomer. In the other irriadiation of the substrate is done in advance in an oxygen atmosphere.

The thrombogenicity of the materials has been evaluated in vitro by exposing the material to fresh whole human blood and measuring the partial thromboplastin time (PTT), Stypven time (ST), and thrombin time (TT) of the exposed blood. With the grafted films cell counts were also usually done. Polyethylene and siliconized glass were used as controls. From the results of the in vitro testing several grafted materials have emerged as promising. Dimethylaminoethyl methacrylate grafts have shown up well in both test systems. 2-sulfoethyl methacrylate grafts and sequential grafts of acrylic acid and 4-vinyl pyridine have both given very good results in one test method. Vinyl pyrrolidone and acrylamide may also be of interest.

Proposed Course:

The contract will be extended. Further optimization of the grafts will be done and an extensive blood compatibility testing program will be developed with the goal of having a material well enough evaluated and characterized to be ready for clinical testing by the end of the year.

Contractor: Rockefeller University

Amount: $52,840

Title: Solute Behavior of Biochemicals Affecting Their Diffusibility Through Cellophane Membranes

Objectives:

This new contract will study the basic parameters affecting the dialysis behavior of a wide variety of solutes and improve further the thin film dialysis apparatus. These parameters will include: molecular weight, pH, solvent system, pore size, protein binding, etc.

Major Findings:

The contractors have determined that the dialysis behavior of molecules is largely a function of molecular size (i.e. volume) and not strictly a function of molecular weight. That is to say a linear molecule of m.w. 400 might have same dialysance as a spherical molecule of weight 2000.

Proposed Course:

They will use nuclear magnetic resonance to elicit further data about the behavior of solutes in solution as it might affect their dialysance.

Contractor: A. J. Sipin Company

Amount: $96,400

Title: Dialysate Delivery System

Objectives:

This contract has as its objectives the development of separate passive fluid resistive elements to meter the flows of the concentrate in water lines on a dialysate delivery system. The pressure drop across the elements is maintained at the same value by a simple concentrate pressuring system. The ratio of the concentrate flow to water flow is in inverse proportion to the resistance of the metering elements.

Major Findings:

Prototypes of this proportioning delivery system have been developed and have been run for some number of hours. The contractor has maintained a constant flow rate for approximately 10 to 14 hours with less than 1% variability in conductivity.

Proposed Course:

The contractor will continue to develop this system toward the goal of a suitcase size portable dialysis unit including a coil container.

Contractor; Southern Research Institute

Amount: $37,943

Title: Activated Carbon Fibers for Use in Artificial Kidney Devices

Objectives:

This is a new contract for work to:

1. Explore the utility of activated carbon fibers for use in artificial kidney devices.

2. Serve as a source of activated carbon fibers for the use of other investigators.

Contractor: Southwest Research Institute

Amount: $30,100

Title: Skin Interfacing Development

Objectives:
This is a new contract to develop a skin cannula junction which will encourage tissue ingrowth and discourage the formation of a sinus tract and will act as a bacteria barrier.

Major Findings:
The investigator has demonstrated that various velour coatings on the outside of cannulas to induce tissue ingrowth were unsuccessful except for one substance, nylon. All cannulae, except the nylon velour coated ones, were extruded from canines. His explanation is that the direction of the nap on the velour may have some effect on its extrusion.

Proposed Work:
The investigator plans to study the effect of fiber orientation in relation to the skin. He will do this by placing the nap in three directions - directed externally, externally and tangential to the cannula surface. He will place nylon collars on half of each of these three groups. He will use pigs this time rather than canines.

Contractor: Stanford Research Institute

Amount: $87,400

Title: Amino Acids, Peptides and Proteoses in Uremia in Man

Objectives: To investigate the amino acid, peptide and proteose composition of uremic plasma, urine, and dialysate and relate it to that found in normal individuals.

Major Findings:

Studies on the Intestinal Absorption of Tryptophan. Their earlier studies of the intestinal absorption of tryptophan in normal subjects and patients have been expanded; they compared results in 10 normal subjects with those in 8 end-stage uremic patients and with those in 6 patients maintained by hemodialysis. At all times, ranging from 30 to 210 minutes following an oral test dose of 25 mg tryptophan/kg, they found that tryptophan levels in patients were about one-half the levels found in normal subjects. Levels in the two groups of patients were not markedly different, showing that maintenance dialysis does not correct the metabolic defect in end-stage uremia that causes the lower levels of tryptophan. In most of the individuals (8 normal subjects and 9 patients), tracer quantities of the nonmetabolized marker amino acid, ^{14}C-cycloleucine, were given simultaneously with the tryptophan dose. All subjects and patients exhibited essentially the same pattern of absorption of the ^{14}C-cycloleucine, showing that no absorption defect for cycloleucine occurs. An assessment of the rate of disappearance of plasma tryptophan in the three groups yielded half-time disappearance values for tryptophan of 1.9 (normal), 1.6 (end-state uremics), and 1.3 hours (dialysis patients). These values suggest slight-to-moderate acceleration of metabolism of tryptophan in the patient groups. The larger difference in plasma levels between the groups compared with the differences in rate of removal of tryptophan suggests that other abnormalities, such as malabsorption, are also present.

Studies on the Binding of Tryptophan by Plasma Proteins. A significant physiologic difference between tryptophan and all other amino acids in plasma is that only tryptophan is bound to plasma proteins. Earlier work by others established that albumin is the protein that binds tryptophan. The investigators earlier work used procedures that deliberately disassociated the tryptophan-albumin complex during deproteinization before analysis and, therefore, had measured total tryptophan--i.e., the sum of protein-bound and unbound tryptophan.

To determine if patients undergoing dialysis differed from normal subjects in the extent of binding of tryptophan by plasma proteins, they employed Amicon membrane filtration cones to obtain ultrafiltrates of

of plasma from 12 normal subjects and 10 uremic patients. The extent of binding was calculated from the tryptophan found before and after ultrafiltration. Albumin concentrations of the plasma were determined concurrently by electrophoresis on cellulose acetate strips. Both total plasma tryptophan and plasma albumin were significantly lower in the patients than in the normal group. The extent of binding of tryptophan in the normal group averaged 80 (± 1) percent, a value similar to that found in normal subjects by others. The mean percentage of tryptophan bound--44 (± 5) percent-- in the patients was significantly lower. In both groups, the extent of binding of tryptophan and the level of plasma albumin were positively correlated. Further examination of the interrelationship among bound and unbound tryptophan and albumin levels was made by plotting the ratio of bound (B) to unbound (UB) tryptophan versus albumin. Both the normal and patient groups showed positive correlations of B/UB tryptophan versus albumin with no overlap between the two groups; but the slopes of the regression lines for the two groups were not different and formed one continuum. This suggests the possibility that not only does tryptophan influence albumin synthesis but also that the lower extent of binding of tryptophan in the patient group may result in accelerated degradation of albumin. It has been reported that tryptophan exerts a protective effect against the proteolysis of albumin when it is complexed with the albumin.

Proposed Course. To determine whether the lower binding of tryptophan in uremic patients is due to an abnormal albumin or to the presence of competing solutes for the tryptophan binding site on albumin.

Contractor: Thomas Jefferson University - Jefferson Medical College

Amount: $18,349

Title: A Clinical Study of Automated Chronic Peritoneal Dialysis

Objectives: To perform a clinical trial of an automated chronic peritoneal dialysis instrument on patients who are unable to be treated by hemodialysis.

Major Findings: A system of automated peritoneal dialysis was developed which retains the simplicity of the standard gravity technique and the safety of a closed system, is capable of overnight unattended dialysis and is financially competitive with home hemodialysis. This system can be conveniently used for in-hospital dialysis but was primarily designed for home hemodialysis by the underprivileged patient who requires chronic dialysis but lacks the trainability for home hemodialysis and the funds for institutional treatment. Twenty-liter plastic containers are filled in the hospital dialysis unit with sterile dialysis solutions by a Seattle peritoneal dialysis sterilizing system. Patients utilize 4 of these a week in two dialysis periods. An attached unit of presterilized tubing and bags are obtained from Cobe Laboratories, Inc., and attached to the automatic cycler which then cycles the 20-liters of dialysis fluid by gravity. The cost of a 40-liter dialysis is $20.

Proposed Course: The objectives of the work having been completed, the contract is being terminated.

Contractor: Tulane University

Amount: $126,000

Title: Studies on the Mechanism of Anemia in Patients with Renal Disease

Major Objectives:

The contractors are attempting to examine hematologic parameters in patients with varying degrees or renal function impairment. Particular emphasis is being placed on the study of erythropoietin, and on renal erythropoietic factor.

Major Finding:

Thirty-five patients have entered this study. Preliminary results indicate that the anemia in these uremic patients consists of a significant degree of marrow depression. A few patients have displayed elevated ESF levels.

Proposed Course:

To continue the study of anemia as it develops over the range of renal excretory function in selected patients with renal disease. To study the acute and chronic effects of hemodialysis on circulating red cells and erythroid function. To determine the erythropoietic response of anemic uremic marrow to erythropoietin and to androgens. To determine the plasma levels of erythropoietin in patients with the anemia of chronic renal disease using a sensitive radioimmunoassay for ESF.

Contractor: University of Utah

Amount: $4,915.00

Title: New Synthetic Membranes for the Dialysis of Blood

Objectives: Gain an understanding of the relationship of the molecular structure of a polymer to its membrane diffusion properties and to its surface interactions with blood.

Major Findings: Characterization of the synthetic block copolymer membranes using a modified Katchalsky approach has been continued. Work has also continued on the interaction of blood constituents and polymer surfaces with emphasis on polymer platelet interactions using the closed cell which was developed by Dr. Lyman for ex vivo investigation. The data developed to date confirmed the direct correlation between platelet adsorption and critical surface tension. Polymer surfaces have been precoated with separate coatings--albumin, gamma globulin, and fibrinogen. The resultant data have demonstrated that albumin-coated polymers tend to be the least thrombogenic from the standpoint of platelet aggregations in the ex vivo test system. They have also characterized membranes from various researchers throughout the country.

Proposed Course: In vivo testing of membrane samples sent from various investigators.

Contractor: University of Utah

Amount: $30,000

Title: A Development of Single Needle Dialysis

Objectives:

Objective of this contract is to develop a device whereby a patient can be dialyzed with the use of a single needle puncture into the arterio-venous fistula rather than the currently common practice of using two needles for dialysis. This is a new contract and has just been initiated and there are no significant advances yet made.

Contractor: University of Utah

Amount: $60,163

Title: Fully Automatic Peritoneal Lavage System Using Reverse Osmosis

Objectives: This is a new contract to develop a safe fully-automatic peritoneal dialysis system using reverse osmosis as the source of water; to determine the relative advantages of peritoneal dialysis on patients who for various reasons may not be suited to maintenance hemodialysis; to test whether pre-pubertal or very small children can be maintained by peritoneal dialysis for prolonged periods and whether daily dialysis of this nature will improve nutritional status, growth, and sexual maturation.

Contractor: University of Utah

Amount: $55,606

Title: Adsorbent Hemoperfusion

Objectives:

To investigate and develop the concept of directly contacting blood on activated carbon for the purpose of removing uremic "toxins".

Major Finding:

In vitro adsorption isotherms have been generated for creatinine, salicylic acid and nembutol. Coating the carbon with glutaraldehyde cross-linked albumin or with polyhydroxyethyl - methacrylate (Hydron) does not significantly reduce adsorption rates or capacities.

In vivo experiments with sheep have shown dramatic creatinine and uric acid clearances through a 10 gram cartridge. Poly-HEMA coated carbon exhibits less platelet depletion than uncoated controls.

Proposed Course:

Charcoal embolization will be investigated in detail by means of autopsy and histopathological studies on acutely treated sheep. Formed element balances will be done and the *in vitro* adsorption studies will be expanded. Chronic experiments will be performed and the possibility of immunologic actions of albumin coatings will be studied.

Contractor: University of Washington

Amount: $155,561

Title: Clinical Hemodialysis Research

Objectives: To develop and clinically evaluate new hemodialysis techniques and theories.

Major Findings:

1. They have demonstrated that lowering dialysate flow rate with a standard Kiil dialyzer from Q_D500 to Q_D100 does not prolong nerve conduction velocity. This result supports the square meter-hour hypothesis (SMHH). In addition they have demonstrated that lowering dialysate flow improves the patients' clotting mechanism. This result suggests that standard dialysis produces a deficiency syndrome. Finally, the patients all report less fatigue and increased well-being on Q_D100 dialysis. Preliminary attempts to put this fact to the test with a double blind evaluation are underway.

2. Six patient-months of experience with a 3-sq. meter hemodialyzer operated for one-third the normal time has demonstrated that the technique is feasible. Surprisingly, reverse urea load and rapid ultrafiltration have not become a limiting factor. Absence of neuropathy supports the SMHH, but it is far too early to draw conclusions.

3. A patient has completed 5 months on the 1/3 sq. meter unit which result tends to refute the SMHH. Another interpretation of this result is that the "middle" molecules are not as large as previously projected and are in the 350 to 800 molecular weight range.

Proposed Course: Efforts will be concentrated on the verification of the square meter-hour hypothesis (SMHH). These include: 1) dialysis at low dialysate flow rates; 2) dialysis with a large (3 sq. meter) hemodialyzer; 3) dialysis with a small surface area hemodialyzer. In addition, they are attempting to develop a double blind dialysis set-up which will permit objective demonstration of the effect of a given alteration in dialysis technique or equipment on patient well-being.

Contractor: University of Washington

Amount: $167,565

Title: Fluid Mechanics, Mass Transfer, and Optimization Studies of Hemodialyzers

Objectives:

1. Engineering support for clinical strategy based on the "Square-Meter-Hour" hypothesis.

2. In vitro and in vivo solute-protein binding studies.

3. Erythrocyte mass transfer studies.

4. Dialyzer performance studies.

5. Mathematical analysis and optimization of large area hollow fiber dialyzer.

Major Finding:

Detailed, mechanistic evaluation of the mass transfer situation in hemodialysis devices has lead to the "Square Meter-Hour" hypothesis and a clinical strategy based on the engineering analysis has been forthcoming. In addition, the effects of solute-protein binding and erythrocyte mass transfer kinetics have been studied for a number of metabolites and drugs.

Proposed Course:

Engineering support for clinical studies based on the "Square Meter-Hour" hypothesis as well as the solute protein binding and erythrocyte mass transfer studies will be continued. In addition, dialyzer performance studies and a mathematical analysis and optimization of the hollow fiber dialyzer will be pursued.

Contractor: University of Washington

Amount: $71,041

Title: Development of a Low Cost Home Peritoneal Dialysis System

Objectives:

Continuing development and evaluation of automated, home peritoneal dialysate supply and delivery systems.

Major Finding:

A system employing an on line reverse osmosis water supply has been evolved and is now undergoing clinical trial in the hospital.

Proposed Course:

Continuing clinical and technical evaluations as well as minor equipment modifications will be pursued, and information which will provide a critical comparison between hemo- and peritoneal dialysis will be sought.

Contractor: University of Washington

Amount: $21,830

Title: Evaluation of Dow Hollow Fiber Artificial Kidney

Objectives:

1,) Determine feasibility of training to use the Dow HFAK in the home.

2.) Determine how safely the Dow HFAK can be reused.

Major Finding:

Out of 10 patients trained to use the HFAK, 3 have perferred to remain on the Kiil. One patient died after two weeks on the HFAK of causes unrelated to the device. The remaining six patients have successfully used the HFAK in the home from 5 to 14 months.

Re-use time has varied from 2 - 5 times to a 40% fiber loss as estimated from a fiber volume determination.

Proposed Course:

"Fiber loss" has not correlated well with solute clearance or ultrafiltration. It is suspected that any of a number of factors may be leading to erroneous fiber volume estimations. The present studies will be supplemented by efforts to develop a more useful fiber loss parameter.

It is desired that reuse of from 5 to 6 times per dialyses can be achieved.

Contractor: University of Washington

Amount: $78,728

Title: The Anemia of Renal Failure

Objectives:

The objective of the contract is to study the anemia of renal failure by using chronically hemodialyzed sheep as the animal model. The following areas will be investigated: (1) oxygen transport mechanism in the normal and anephric state; (2) the relationship of erythropoietin and marrow production to changes in oxygen delivery in the normal and anephric state; (3) the effect of chromic hemodialysis on erythropoietin function and oxygen transport in the anephric state; (4) the role of inhibition of marrow function in uremic state.

Major Findings:

Preliminary data has been accumulated on the quantitation of the oxygen transport mechanism in the normal state on the relationship of ESF and marrow production to changes in oxygen delivery in normal and anephric state, and on the role of inhibition of marrow function in the uremic state.

Proposed Course:

To continue gathering data on the various factors contributing to the anemia of renal failure.

Contractor: The University of Washington

Amount: $152,100

Title: Cannula Research for Hemodialysis

Objectives:
Investigate by means of a broad approach improvement of an access to the circulation.

Major Findings:
The sheep has been evaluated as a model for cannula testing. The larger bore AV cannula has been adequately developed and has proven to be useful in that a higher flow can be achieved through it. The Dacron applique shunt has not been used recently because of recurrent infection problems with at least one fatality associated with this. The problems of infection prevention are currently being studied in the Dacron applique shunt. Dipyridamole has been found to apparently inhibit platelet aggregation *in vivo*.

Proposed Course:
Continue the current objectives utilizing the animal shunt model to assess the effects of various designs and drugs on cannula longevity.

Contractor: University of Washington

Amount: $30,536

Title: Acid-Base Chemistry and Human Bone

Objective: The new contractor will study the buffering capacity of bone by evaluation of the lobile carbonate fraction in uremic, acidatic patients.

THE ANNUAL REPORT SUMMARY
EPIDEMIOLOGY AND FIELD STUDIES BRANCH

SOUTHWESTERN FIELD STUDIES SECTION

The Phoenix office of the Southwestern Field Studies Section was relocated in December 1971 to office space closer to the Phoenix Indian Medical Center. This change was necessitated by the expiration of a lease, but resulted in a reallocation of area to conform more closely to the program requirements of the Section.

In September 1971, Mrs. Anne Kious, senior computer programmer to the Section, resigned after three years of service. Her resignation has had a major impact on the productivity of the Section during the past year, as her position could not be refilled because of the personnel freeze. This restriction has damaged the development of the program of the Section, as necessary analyses have been unavailable since the retrieval of virtually all data collected during the past six years is dependent upon the availability of sufficient computer programming personnel.

A paper on Retinopathy in the Pima Indians, by Dr. Arthur Dorf, Staff Associate, has been selected a finalist for the 1972 J. D. Lane award of the Commissioned Officers Association and Clinical Society of the U. S. Public Health Service.

Diabetes Mellitus

The major program activity of the Section has remained the epidemiologic investigation of diabetes mellitus among Indians of the Southwestern United States, and in particular the Pima Indians of Arizona.

The relationship of retinopathy and nephropathy to duration of diabetes mellitus has indicated that, although the frequency of these complications increases with duration, there appears to be an appreciable number of subjects with such complications at the time of discovery of glucose intolerance. This, together with the observation that among those with newly discovered glucose intolerance the frequency of such complications is unrelated to the degree of carbohydrate abnormality, strongly suggests that such complications may be independent of the development of hyperglycemia. If so, this implies that both phenomena have a common basis and that the development of the specific complications would be unlikely to be influenced by changes in the circulating glucose level.

Analysis of the relationship of blood pressure to retinopathy in the diabetic has indicated that the incidence of this complication is a function of the blood pressure level. It seems possible that control of blood pressure in the diabetic subject may reduce the incidence of diabetic

retinopathy. This hypothesis requires testing in a controlled therapeutic trial. Because of the close interrelationship of nephropathy and retinopathy it would not be surprising if the renal disease of diabetes were also influenced in a similar manner.

The insensitivity and lack of specificity of the conventional measurements of renal function in diabetes severely hamper assessment of this condition and prevent anything other than a crude staging of the disease. Because of the high prevalence of the specific changes of diabetic nephropathy seen at autopsy, the Pima Indian population appears an ideal group in whom to characterize the protein excretion, both quantitatively and qualitatively, in the earliest detectable stages of diabetic nephropathy. The development of methodology which may be applied to this problem has been rapid in recent years and consequently a detailed investigation of this problem is planned.

Arthritis

The detailed analysis of two sets of criteria proposed for the diagnosis of rheumatoid arthritis in population studies has indicated some major problems of specificity, especially as these relate to one set of criteria. Analysis of data collected in the Pima Indians has suggested that a simple modification of this set of criteria may result in a much higher degree of specificity than by use of either set as originally proposed.

The development of such criteria remains an important goal, since the criteria form a basis for the investigation of the distribution and determinants of rheumatoid arthritis in population studies.

Gallbladder Disease

The establishment of the patho-physiologic basis of cholesterol gallstone formation in the American Indian and other populations as the result of diminished bile salt secretion, has led to the realization that the prevention and dissolution of gallstones by the oral administration of bile salts may be possible.

The suitability of the Pima Indian population for the demonstration of the potential of this mode of therapy has led to the design of a clinical trial to determine the ability of cholic and chenodeoxycholic acid to dissolve asymptomatic gallstones. This trial will be implemented as soon as possible.

METABOLIC DISEASES EPIDEMIOLOGY UNIT

The interests of the Unit have continued to center around the identification of goitrogenic substances which might be responsible for the high prevalence of endemic goiter in certain areas. In addition, the identification of adventitious sources of iodide has been continued.

A large number of micro-organisms obtained from well water in an area of high endemicity for goiter have been screened for goitrogenic activity in the rat. The major findings have indicated that several organisms, Penicillium species and the Desulfovibrio desulfuricans, are particularly likely to produce goitrogenic products, which may be responsible for the development of goiter in areas of contaminated water supply.

Further work has confirmed that the average daily intake of iodine in the American diet is approximately 400 µg. One of the sources of this apparently excessive intake has been shown to be erythrosine, a coloring matter used in a large variety of food and pharmaceutical products.

Further isolation studies and testing of goitrogenic activity from these isolates will be continued.

Serial No. NIAMD-EFS-1(c)
1. Epidemiology and Field Studies Branch
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Prevalence of Diabetes in Indian Populations in the Southwestern United States

Previous Serial Number: Same

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Miss Cheryl Ritenbaugh

Cooperating Units: Indian Health Service (PHS)

Man Years:

Total:	0.6
Professional:	0.3
Others:	0.3

Project Description:

Objectives:

1. To determine the prevalence of diabetes among various native populations in the Southwestern United States.

2. To formulate and test hypotheses which may explain the high frequency of diabetes mellitus among some of these populations.

Methods:

Random samples of total populations will be examined by means of standardized tests of glucose tolerance to enable the prevalence of glucose intolerance to be determined. Other examinations will be performed to document the frequency of other disorders when appropriate.

Major Findings:

A survey was conducted to determine the frequency of glucose intolerance among the Navajo Indians residing in the Fort Defiance area of the Navajo Indian Reservation. The methods used were identical to those used in the other studies cited below. The Navajo, among whom diabetes has been said to be rare, belong to the Athabascan linguistic group, and are thus rather closely related to the Apache Indians.

PREVALENCE OF HYPERGLYCEMIA* AMONG SOUTHWESTERN INDIANS

	Males				Females			
Age (years)	<35		≥35		<35		≥35	
Tribe	No. Exam.	Percent Positive	No. Exam.	Percent Positive	No. Exam.	Percent Positive	No. Exam.	Percent Positive
Navajo	21	0.0	28	7.1	36	2.8	101	8.9
White River Apache	--	--	109	3.7	--	--	159	18.2
Washoe	16	0.0	34	8.8	30	3.3	32	25.0
Paiute	39	0.0	27	18.5	38	2.6	27	33.3
Upland Yumans	52	0.0	144	21.2	93	1.8	169	38.6
Zuni	87	2.9	140	17.2	312	3.8	152	45.4
Cocopah	15	6.7	35	25.7	29	6.9	44	40.9
Pima	921	4.3	437	41.0	1098	6.5	461	58.1

*Plasma glucose level ≥160/100ml two hours post 75G carbohydrate load or ≥180mg/100ml one hour post load.

Significance of Research:

The prevalence rates of diabetes mellitus among the various Indian tribes in the Southwestern United States appear to be substantially unrelated to their genetic origins. This finding suggests that the high prevalence of diabetes observed in many may represent an adaptation, with a possible genetic basis, to certain types of environment, rather than simply an expression of a common genetic background.

Proposed Course:

Glucose tolerance testing will be continued among other Indian tribes residing in the Southwestern United States in order to define further the possible reasons for the great variation in the prevalence of diabetes mellitus in this area.

Honors and Awards: None.

Publications:

Bennett, P.H. and Miller, M.: Diabetes Mellitus in Indians of the Southwestern United States. In Rodriguez, R.R. and Vallance-Owen, J. (Eds): Diabetes, Proceedings of the Seventh Congress of the International Diabetes Federation (Buenos Aires 1970), International Congress Series No. 231. Amsterdam, Excerpta Medica, 1971, pp. 318-324.

Serial No. NIAMD-EFS-2(c)
1. Epidemiology and Field Studies Branch
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Prospective Study of the Natural History of Diabetes Mellitus in the Gila River Indian Community

Previous Serial Number: Same

Principal Investigator: Dr. Peter H. Bennett

Other Investigators:
Dr. Arthur Dorf
Dr. S. A. Kamenetzky
Dr. J. A. Ingelfinger
Miss P. E. Sperling
Dr. Max Miller
Dr. Arthur G. Steinberg
Dr. Norman B. Rushforth
Dr. Elmer J. Ballintine
Dr. Irving M. Liebow
Dr. Frederick A. Rose
Dr. James E. Maynard

Cooperating Units:
Indian Health Service (PHS)
Case Western Reserve University
Center for Disease Control (PHS)

Man Years:

Total:	9.6
Professional:	3.6
Others:	6.0

Project Description:

Objectives:

1. Determine the prevalence and incidence of chemical and clinical diabetes mellitus among those five years of age and over on the Gila River Indian Reservation.

2. Evaluate factors associated with the progression from prediabetic state to chemical diabetes mellitus and to clinical diabetes.

3. Study the association between diabetes and rate of appearance and progression of vascular complications.

4. Investigate influence of tribe, age, obesity, parity, and other factors on changes in glucose tolerance and their relationship to diabetes mellitus.

Methods:

1. The following tests and examinations will be repeated at two yearly intervals on all Indians five years of age or more living on the Sacaton Service Unit portion of the Gila River Reservation:

a. Clinical interview
b. Physical examination
c. Modified glucose tolerance test
d. Fundus photographs
e. Electrocardiogram
f. Tests for vibratory sensation threshold
g. Soft tissue radiographs for vascular calcification

2. Some of the above tests and examinations will also be done on Indians of other tribes or living under different environmental conditions for comparison.

Major Findings:

1. Retinopathy and Renal Disease: Retinopathy was more prevalent in male and female diabetics with proteinuria ≥30mg/100ml, than among those with less or no proteinuria. No significant difference was found in the prevalence of retinopathy between male and female diabetics with proteinuria ≥30mg/100ml. Among non-diabetics, subjects with no history of glucose intolerance whose two hour post-load plasma glucose level was <200mg/100ml, retinopathy and proteinuria were not related.

PERCENT OF SUBJECTS WITH RETINOPATHY
AND THEIR RELATIONSHIP TO PROTEINURIA

Proteinuria	Males		Females		Both Sexes
	No. Exam.	Percent Positive	No. Exam.	Percent Positive	Percent Positive
With Diabetes					
Absent	94	22.3	151	21.8	22.0
≥30mg/100ml	20	50.0	35	54.3	52.7
Without Diabetes					
Absent	353	5.7	402	5.7	5.7
≥30mg/100ml	25	4.0	28	0.0	1.9

Quantitative estimates of proteinuria were made in 49 of the 55 diabetic subjects with proteinuria by measuring the albumin and creatinine concentrations in the two hour urine samples. Diabetic males and females with higher albumin/creatinine (A/C) ratios had a significantly higher frequency of retinopathy than those with lower A/C ratios. Among diabetic subjects whose urine specimen was positive for protein, $\geq$30mg/100ml by dipstick analysis, only 30 percent of those who excreted less than 1 gram protein per day had retinopathy. Among those who excreted 1.0-4.9 grams protein per day, 50 percent had retinopathy and retinopathy was found in more than 90 percent of those who excreted 5 grams or more protein per day (χ^2=11.2; $p<0.01$). Thus increased severity of renal disease--as determined by severity of proteinuria--was associated with an increased prevalence of retinopathy.

Since the frequency of proteinuria, like the frequency of retinopathy, increased with increasing duration of diabetes, it was necessary to stratify for duration of diabetes to determine whether the relationship between retinopathy and proteinuria was due to the relationship of each to duration of diabetes. Apart from the "newly diagnosed", where there was no relationship of the frequency of retinopathy to the presence of proteinuria, retinopathy was more prevalent among those diabetics with proteinuria regardless of duration of diabetes. Subjects with proteinuria and with duration 0-4 years, 5-9 years, and 10 years or more had nearly twice the frequency of retinopathy than subjects with similar durations of diabetes who had no proteinuria.

PERCENT OF DIABETIC SUBJECTS WITH RETINOPATHY
AND THEIR RELATIONSHIP TO PROTEINURIA AND DURATION OF DIABETES

	Duration of Diabetes							
Proteinuria	Newly Diagnosed		0-4 Years		5-9 Years		$\geq$10 Years	
	No. Exam.	Percent Positive	No. Exam.	Percent Positive	No. Exam.	Percent Positive	No. Exam.	Percent Positive
Absent	76	13.2	92	22.8	49	24.5	28	22.0
$\geq$30mg/100ml	13	7.7	12	58.3	10	60.0	20	52.7

Among diabetic subjects, the frequency of each element of retinopathy also increased with increasing serum creatinine concentrations.

PERCENT OF DIABETIC SUBJECTS WITH RETINOPATHY
AND THEIR RELATIONSHIP TO SERUM CREATININE LEVELS

Serum Creatinine (mg/100ml)	Males		Females		Both Sexes
	No. Exam.	Percent Positive	No. Exam.	Percent Positive	Percent Positive
0.0-0.9	80	22.5	147	23.8	23.4
1.0-1.4	26	34.6	23	47.8	40.8
1.5+	5	60.0	6	83.3	72.7

Retinopathy was more frequent among diabetics with elevated serum creatinine levels, regardless of duration of diabetes.

PERCENT OF DIABETIC SUBJECTS WITH RETINOPATHY
AND THEIR RELATIONSHIP TO SERUM CREATININE LEVELS AND DURATION OF DIABETES

Serum Creatinine (mg/100ml)	Duration of Diabetes: Less than 10 Years No. Exam.	Less than 10 Years Percent Positive	10 Years or More No. Exam.	10 Years or More Percent Positive
0.0-0.9	195	18.5	32	53.1
1.0-1.4	39	35.9	10	60.0
1.5+	4	75.0	7	71.4

2. Blood Pressure and Retinopathy: The prevalence of retinopathy increased with increasing systolic blood pressure in diabetic males and females. Retinopathy was found in 13 percent of those with systolic blood pressures <140 mmHg, in 29 percent of those with systolic blood pressures of 140-159 mmHg, and in 49 percent of those with systolic blood pressures ≥160 mmHg ($\chi^2>30.0$; $p<0.001$).

Among non-diabetics, the prevalence of retinopathy increased somewhat with increasing systolic blood pressure, but this relationship was largely due to the relatively high prevalence of exudates among those with systolic blood pressure ≥160 mmHg. This finding might have been anticipated since no attempt was made to distinguish between the types of exudate. More important, however, is the fact that all other elements of retinopathy were rare and failed to show a relationship to blood pressure in the non-diabetic indicating that the positive relationship found among diabetics was not due to the occurrence of hypertensive retinopathy per se.

Retinopathy was more frequent in diabetics with elevated systolic blood pressures regardless of duration of diabetes. Those with systolic blood pressure of <140 mmHg maintained a relatively low prevalence of retinopathy regardless of duration of diabetes; after duration of 10 years or more, less than one-fifth had retinopathy. Conversely, diabetics with systolic blood pressure ≥160 mmHg were found to have a steeply increasing frequency of retinopathy with increasing duration of diabetes; of those with duration of 10 years or more, 85 percent had retinopathy.

PERCENT OF DIABETIC SUBJECTS WITH RETINOPATHY
IN RELATIONSHIP TO BLOOD PRESSURE AND DURATION OF DIABETES

Blood Pressure (mmHg)	Duration of Diabetes: Newly Diagnosed No. Exam.	Newly Diagnosed Percent Positive	0-4 Years No. Exam.	0-4 Years Percent Positive	5-9 Years No. Exam.	5-9 Years Percent Positive	≥10 Years No. Exam.	≥10 Years Percent Positive
Systolic								
0-139	46	8.7	52	13.5	23	17.4	11	18.2
140-159	19	10.5	25	32.0	16	18.8	19	52.6
160+	27	18.5	29	48.3	20	55.0	20	85.0
Diastolic								
0-79	34	8.8	41	19.5	18	22.2	18	38.9
80-99	45	11.1	55	32.7	32	28.1	24	58.3
100+	13	23.1	10	30.0	9	55.6	8	100.0

In order to determine whether blood pressure might be causally related to the development of retinopathy in the diabetic subject, the incidence of microaneurysms was measured in subjects, aged 25-64, who demonstrated no retinopathy at the baseline examination. After four years of follow-up, diabetic subjects with baseline blood pressure of ≥140 mmHg had twice the frequency of microaneurysms as subjects with baseline systolic blood pressure <140 mmHg. This relationship was significant ($\chi^2=6.07$; $p<0.05$).

RELATIONSHIP OF BASELINE SYSTOLIC BLOOD PRESSURE TO THE INCIDENCE OF MICROANEURYSMS AMONG DIABETIC SUBJECTS, AGED 25-64, WITH NO RETINOPATHY AT BASELINE EXAMINATION

Baseline Systolic B.P.(mmHg)	No. Exam.	Average Age at Baseline (yrs)	Average Duration of Diabetes at Baseline (yrs)	Average Duration of Follow-up (yrs)	Percent Positive
0-139	80	42.3	2.56	3.98	16.2
140-159	38	47.9	3.55	3.84	34.2
160+	27	47.6	2.85	3.97	33.3

The present findings suggest that blood pressure may be a factor in the pathogenesis or progression of retinopathy among diabetics. This hypothesis is supported by the fact that the incidence of microaneurysms was related to baseline systolic blood pressure.

Retinopathy and renal disease were closely related among diabetic Pima Indians. The prevalence of retinopathy was related to proteinuria and elevated serum creatinine levels. These relationships were not affected by

sex or duration of diabetes, though retinopathy, proteinuria, and elevated serum creatinine levels each increased in frequency with increasing duration. These relationships among diabetics are consistent with the syndrome resulting from diabetic microangiopathy described in other populations.

3. Renal Disease: Among the Pima Indians, renal disease is responsible for 14 percent of all non-traumatic deaths, a rate approximately five times greater than that for the general United States population. Because of the high prevalence of diabetes mellitus in the population, the relationship between carbohydrate intolerance and kidney disease was investigated. Modified glucose tolerance tests, urine protein concentrations (estimated by dipstick and quantified by the albumin/creatinine (A/C) ratio), and serum creatinine determinations were performed in over 1600 half- to full-blooded Pima Indians aged 15 and over.

Proteinuria ($\geq$30mg/100ml) occurred in 22 percent of the diabetics and six percent of non-diabetics ($p<.001$). No sex difference in the prevalence of proteinuria in either diabetics or non-diabetics was observed. Among diabetics of every age group, the frequency of proteinuria was greater than in non-diabetics, and more severe degrees of proteinuria (A/C ratio $\geq$1.0) were 16 times more prevalent in the diabetics. Both the frequency of proteinuria, and the severity increased with increasing duration of diabetes.

In the non-diabetics with systolic hypertension ($\geq$160 mmHg), there was no increase in the frequency of proteinuria. In contrast, in the diabetics who were under age 65 at the time of examination, proteinuria was significantly more prevalent in those with systolic hypertension. Similar trends were noted in persons with diastolic hypertension ($\geq$100 mmHg) but the differences were not statistically significant.

Two-thirds of those with elevation of the serum creatinine ($\geq$1.5mg/100ml) were diabetic, and elevated levels were twelve times more common in the diabetics than in non-diabetics ($p<.001$). Urinary A/C ratios $\geq$1.0 were present in 85 percent of those with elevated creatinines. The percentage of persons with elevation of the serum creatinine increased with increasing duration of diabetes. The mean duration of diabetes of those with this abnormality was 10 years.

RELATIONSHIP OF DURATION OF DIABETES TO ELEVATED SERUM CREATININE LEVEL ($\geq$1.5mg/100ml)

	Duration of Diabetes							
Sex	Newly Diagnosed		0-4 Years		5-9 Years		$\geq$10 Years	
	No. Exam.	Percent Positive	No. Exam.	Percent Positive	No. Exam.	Percent Positive	No. Exam.	Percent Positive
Males	49	0.0	59	6.8	31	6.5	23	8.7
Females	66	0.0	84	0.0	50	6.0	41	19.5
Both Sexes	115	0.0	143	2.8	81	6.2	64	15.6

4. Microvascular Complications in Early Diabetes: Since it is unclear whether the microangiopathy of diabetes mellitus occurs secondary to hyperglycemia or represents an alternative manifestation of a more basic metabolic defect, the frequency of retinal and renal disease and its relationship to glucose tolerance among subjects without previously recognized glucose intolerance was examined.

FREQUENCY OF RENAL AND RETINAL DISEASE IN PIMA INDIANS
WITHOUT PREVIOUSLY RECOGNIZED GLUCOSE INTOLERANCE

Two hour plasma glucose (mg/100ml)	Renal Disease			Retinopathy		
	No. Exam.	Percent Proteinuria	Percent A/C>1.0	No. Exam.	Percent with Retinal changes	
50- 99	427	4.2	0.6	260	3.5	
100-125	465	7.1	1.0	325	5.8	
126-159	287	5.6	1.2	175	4.6	4.9
160-199	103	8.7	1.5	71	7.0	
200-250	56	17.9	9.0	40	10.0	
251-315	17	17.6	11.7	13	15.4	
316-399	23	21.7	10.9	13	7.7	12.0
400-500	22	9.1	9.1	16	25.0	
501+	16	12.5	12.5	10	0.0	
Total	1416	6.9		923	5.6	

Among these subjects, complications were found much more frequently in those subjects who had two hour post-load plasma glucose levels of 200mg/100ml and over. This observation suggests that the microvascular complications of diabetes may be found concurrently with the discovery of hyperglycemia and does not preclude the possibility that such complications might precede the development of glucose intolerance in some subjects. It was also observed that among those with two hour plasma glucose levels in excess of 200mg/100ml, that the frequency of microvascular complications was unrelated to the glucose level, suggesting that in the diabetic, the presence or absence of microangiopathy is unrelated to the degree of glucose intolerance.

Both observations are consistent with the hypothesis that the microangiopathy of diabetes mellitus and hyperglycemia arise independently from a more basic abnormality.

5. Coronary Heart Disease: Twelve lead electrocardiograms from 702 Pima Indians, age 40 years and over, were read according to the Minnesota code and the rates of coronary heart disease (abnormal Q waves, ST and T wave changes, left bundle branch or complete heart block) were compared to those reported from the predominantly white population of Tecumseh, Michigan.

PREVALENCE OF ELECTROCARDIOGRAPHIC CHANGES*
IN PIMA INDIANS AND POPULATION OF TECUMSEH, MICHIGAN

Age	PIMA Non-Diabetic		PIMA Diabetic		TECUMSEH	
	No. Exam.	% with Changes	No. Exam.	% with Changes	No. Exam.	% with Changes
Males						
40-59	125	1.6	76	1.3	796	5.3
60+	95	4.2	55	5.5	301	17.3
Females						
40-59	117	0.0	124	2.4	797	2.4
60+	48	0.0	72	7.0	354	12.4

* Q - Minnesota code I 1 or 2;
ST-T - Minnesota code IV 1 and V 1 or 2;
LBBB or CHB - Minnesota code VI 1 or VII 1.

The age-sex standardized prevalence of CHD in the Tecumseh population (7.3 percent) was three times greater than in the Pima Indians (2.3 percent; $p<0.001$). This population difference was found in both males (9.1 percent vs. 2.5 percent; $p<0.001$) and females (5.5 percent vs. 2.2 percent; $p<0.025$).

Both male and female Pima Indians with diabetes had rates of CHD which were less than those observed in the corresponding age and sex groups in the general Tecumseh population, but the difference was significant only for the males ($p<0.05$).

Although CHD was more prevalent in the Pima diabetics compared to the non-diabetics (3.5 percent vs. 1.3 percent), no significant difference was found between the male diabetic and non-diabetic subjects (2.9 percent vs. 2.6 percent). In the females, however, CHD was found in 8 of the 196 diabetic subjects, but in none of the 165 non-diabetic subjects ($p<0.01$).

The electrocardiograms of 1154 Pima Indians were read according to the Minnesota code. Left Axis Deviation was more prevalent in the diabetic Pima than in the non-diabetic Pima.

LEFT AXIS DEVIATION
(Rate/100)

Age	25-34	35-44	45-54	55-64	65-74	75+
Males						
Non-Diabetic	2.1	7.0	8.6	11.7	21.4	14.8
Diabetic	4.3	11.3	16.1	22.5	32.0	18.1
Females						
Non-Diabetic	1.3	3.6	6.1	3.0	14.8	0
Diabetic	3.0	1.5	3.7	12.6	25.9	0

This difference is not significant. However, the diabetics with Left Axis Deviation had significantly more diastolic hypertension than diabetics of similar age and sex without Left Axis Deviation. Non-diabetics with Left Axis Deviation had no more diastolic hypertension than age matched non-diabetic controls.

6. Normal Serum Creatinine Levels: The normal limits of serum creatinine among the Pima Indians have been determined by age and sex.

NORMAL VALUES FOR SERUM CREATININE (mg/100ml) IN PIMA INDIANS

Age Groups	MALES No. Exam.	Mean $\bar{x}$	Std.Dev.	Upper Limit of Range*	FEMALES No. Exam.	Mean $\bar{x}$	Std.Dev.	Upper Limit of Range*
5-9	251	0.45	0.079	0.61	262	0.43	0.073	0.67
10-14	192	0.53	0.119	0.77	219	0.51	0.113	0.74
15-24	198	0.72	0.155	1.03	243	0.55	0.12	0.79
25-34	86	0.75	0.163	1.07	115	0.56	0.16	0.80
35-44	77	0.80	0.142	1.08	79	0.63	0.14	0.91
45-54	43	0.83	0.173	1.17	37	0.63	0.16	0.96
55-64	40	0.86	0.193	1.25	24	0.70	0.15	1.00
65+	47	0.91	0.187	1.28	27	0.73	0.14	1.01

* $\bar{x}$ + 2 S.D.

These data will be used to describe the frequency of renal failure among the Pima as part of a more intensive program to evaluate the types of renal abnormality, their frequency, and their association with diabetes among the Pima.

7. Evaluation of One-Hour and Two-Hour Glucose Tolerance Test: There has been considerable debate concerning the relative merits of the one-hour post-load glucose determination and the two-hour determination as the optimal method of screening for diabetes mellitus.

Plasma glucose levels one and two hours after a glucose load were determined in over 1600 Pima Indians, aged 16 years and over. Both one- and two-hour levels gave frequency distributions consistent with a model of two overlapping Gaussian distributions.

The amount of overlapping of distributions, however, was greater for the one-hour than the two-hour values. Levels were determined which minimized the sum of the probabilities of misclassification, that is, of classifying a diabetic as normal, and vice versa, for each glucose value. In each age-sex group, the probabilities of misclassification were less for the two-hour glucose level, 45% less in males and 30% less in females.

The findings provide a good rationale for preferring the two-hour glucose value for the screening and diagnosis of diabetes.

8. Coxsackie B_4 Virus Studies: The previously reported studies on Coxsackie B_4 virus antibody and their relationship to diabetes mellitus of recent onset have been extended. Thirty-three pairs of sera, one of each pair from a newly diagnosed diabetic and the other from an age, sex matched control subject sampled contemporaneously, have been examined.

In five pairs, the titer in the diabetic subject was $\geq$1:64 compared to one pair in whom the control subject equalled this level (χ^2=2.7).

Although not statistically significant, the strength of the association between the Coxsackie B_4 antibody level and the presence of diabetes is such that this study will be further extended.

Significance of Research:

The vascular complications of diabetes mellitus are responsible for the majority of the morbidity and mortality associated with diabetes mellitus. The relationship of hypertension to retinopathy in the diabetic appears to represent an important factor in the pathogenesis of the retinal changes, and one whose effect is potentially amenable to alteration by safe medical means.

The observation of the close association of renal disease and retinal abnormalities suggests that factors influencing the progression of one of these complications might well influence the other. However, the presently applied methods of investigation for renal disease are relatively insensitive and non-specific, and it seems probable that the application of more quantitative, sensitive, and discriminating methods for the evaluation of renal function could lead to a better understanding of factors related to the development and progression of diabetic nephropathy.

The observation of a high prevalence of eye and kidney abnormalities in subjects with newly recognized glucose intolerance suggests that a

more basic abnormality than simply hyperglycemia is responsible for the development of the vascular lesions.

Proposed Course:

The prospective study of diabetes and its complications will be continued. The frequency and characteristics of renal functional abnormalities will be determined using sensitive methods for the measurement and characterization of proteinuria and applied to subjects with subclinical evidence of renal disease by conventional definitions, i.e., subjects without proteinuria. The early recognition and natural history of proteinuria in the diabetic may lead to the recognition of important modifying factors.

The blood lipids of the Pima Indian population will be characterized to determine if their low prevalence of coronary heart disease is related to unusual lipid characteristics.

Additional studies will be performed to document the effects of Coxsackie B_4 virus infection on glucose tolerance. Since animal studies have indicated that this organism will selectively destroy the pancreatic beta cell it is hoped to document the incidence of diabetes following Coxsackie B_4 infection in a controlled manner among the Pima Indians.

Since a large number of prediabetic subjects have now been identified additional analyses will be performed to try to identify any factors which lead to the development of diabetes mellitus in this high risk group.

Honors and Awards: None.

Publications:

Bennett, P.H., Burch, T.A., and Miller, M.: Diabetes mellitus in American (Pima) Indians. Lancet ii: 125-128, 1971.

Bennett, P.H., Burch, T.A., and Miller, M.: The high prevalence of diabetes in the Pima Indians of Arizona, U.S.A. In Tsuji, S. and Wada, M. (Eds.): Diabetes Mellitus in Asia, 1970, Proc. of a Symposium, Kobe, Japan, 24 May 1970. International Congress Series No. 221. Amsterdam, Excerpta Medica Foundation, 1971, pp. 33-39.

Bennett, P.H. and Miller, M.: Diabetes in the Pima Indians. Letters to the Editor. Lancet ii: 488-489, 1971.

Rushforth, N.B., Bennett, P.H., Steinberg, A.G., Burch, T.A., and Miller, M.: Diabetes in the Pima Indians: Evidence of bimodality in glucose tolerance distributions. Diabetes 20: 756-765, 1971.

Bennett, P.H.: Diabetes in the desert--the Pima Indians of Arizona. ADA Forecast. In press.

Bennett, P.H., and Miller, M.: The microvascular complications of early diabetes in the Pima Indian. In Proceedings of the II International Symposium on Early Diabetes, Curacao, December 1971. In press.

Serial No. NIAMD-EFS-3(c)
1. Epidemiology and Field Studies Branch
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Prospective Study of the Natural History of Arthritis and Rheumatism in the Gila River Indian Community

Previous Serial Number: Same

Principal Investigator: Dr. Peter H. Bennett

Other Investigators:
Dr. J.-C. Henrard (Guest Worker)
Dr. Thomas A. Burch
Dr. Arthur Dorf
Dr. S. A. Kamenetzky
Dr. J. A. Ingelfinger
Miss P. E. Sperling

Cooperating Units:
Indian Health Service (PHS)
Hawaii State Health Department

Man Years:

Total:	2.0
Professional:	1.0
Others:	1.0

Project Description:

Objectives:

1. Determine the progression of clinical, radiological and serological features of joint diseases affecting the Gila River Indians with particular attention to rheumatoid arthritis, osteoarthrosis, ankylosing spondylitis, and gout.

2. Determine environmental and genetic factors associated with development and progression of arthritis and rheumatism.

3. To compare and contrast the clinical course of rheumatoid arthritis in sero-positive individuals with that of sero-negative arthritics.

4. Determine the predicative and prognostic significance of rheumatoid factor in relation to the clinical course of rheumatoid arthritis.

Methods:

1. The following tests and examinations will be repeated on all Indians five years of age or over, living on the Sacaton Service Unit portion of the Gila River Reservation.

a. Clinical interview.
b. Physical examination.
c. Radiographs of hands, feet, and cervical spine. Males over age 14 and females over age 44 will have a pelvis film taken.
d. Laboratory determinations: rheumatoid factor, uric acid.

2. The data from the above examinations and tests as well as demographic, pedigree, environmental information, and data on other conditions and diseases will also be collected.

Major Findings:

Rheumatoid Arthritis: The utility and value of two sets of criteria for the diagnosis of rheumatoid arthritis in population surveys were compared in data collected among over 1700 Pima Indians aged 15 years and over.

The overall prevalence of probable and definite active R.A. did not differ significantly when graded by C.I.O.M.S. criteria (5.2 percent) or by New York criteria (5.9 percent). Using the C.I.O.M.S. criteria, a slightly higher prevalence of R.A. was found among females (6.0 percent vs. 4.3 in males; sex ratio 0.7) whereas, using the New York criteria a higher prevalence was observed in males (6.7 vs. 5.3 percent in females; sex ratio 1.3). The difference in ratios was significant ($p<0.05$).

Less than 30 percent of persons positive by one set of criteria were so by the other; 20 percent of those having R.A. by C.I.O.M.S. criteria had none of the New York criteria, and 60 percent of those having R.A. by New York criteria had less than two C.I.O.M.S. criteria. The differences were due in part to a higher frequency of limitation of motion in elbows and M.C.P. joints in males resulting in many who were positive by the second New York criterion, and partly to a higher frequency of soft tissue swelling of knees and ankles in females resulting in many who scored up to three points for joint swelling in the C.I.O.M.S. clinical criteria.

In order to correct some of the apparent weakness of the New York criteria while still preserving the weight attributable to the pattern of joint involvement, which is an important feature of R.A., the second New York criterion was applied after excluding limitation of joint motion as one of its components. The overall prevalence rate obtained was 3.0 percent with a predominance among the females (3.8 vs. 2.0 percent in males), results approximating more closely to those of other population surveys. The modified criteria appeared to identify subjects in whom the experienced clinical rheumatologist would more often agree with the diagnosis.

Significance:

The epidemiology of rheumatoid arthritis has been hampered by the difficulty in specific diagnosis because of the protean manifestations of the disease. The present findings suggest that additional modifications of diagnostic criteria are required if both sensitive and specific indices of the disease are to be defined. Definition of the disease seems a prerequisite for the exploration of its distribution and determinants.

Proposed Course:

The collection of data from the Pima Indians at biennial intervals will continue so that the development and progression of various rheumatic diseases may be observed.

The radiographs collected over the past five years have been evaluated on a joint by joint basis and these readings will be subjected to "pattern" analysis to determine if previously unrecognized syndromes of degenerative and erosive arthritis can be identified.

Honors and Awards: None.

Publications:

Sperling, P.E.: Diluter modified to specific need for serial dilutions. Amer. J. Med. Tech. 37: 24, 1971.

Gofton, J.P., Bennett, P.H., Smythe, H.S., and Decker, J.L.: Sacroiliitis and ankylosing spondylitis in North American Indians. Ann. Rheum. Dis. In press.

Serial No. NIAMD-EFS-4(c)
1. Epidemiology and Field Studies Branch
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Gila River Indian Community Gallbladder Study

Previous Serial Number: Same

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Dr. J. A. Ingelfinger
Dr. S. M. Grundy
Dr. M. P. Tyor
Dr. Donald M. Small

Cooperating Units: Indian Health Service (PHS)
Boston University

Man Years:

Total:	1.0
Professional:	0.5
Other:	0.5

Project Description:

Objectives:

To determine whether cholesterol gallstones may be dissolved by the oral administration of bile salts.

Methods:

1. Adult Pima Indian subjects residing on the Gila River Indian Reservation who have asymptomatic gallstones will be identified.

2. Such subjects will be asked to participate in a double blind controlled study of the effect of oral administration of cholic and chenodeoxycholic acid in producing the dissolution of the gallstones over a two-year period.

Major Findings:

None. All activity in this project so far has been directed towards the development of a detailed study protocol. It is expected that recruitment of participants will commence in the coming year.

Significance of Research:

The metabolic basis of cholesterol gallstone formation, a diminished bile salt secretion rate in association with a normal or elevated biliary cholesterol secretion, which leads to the production of a cholesterol-supersaturated (lithogenic) bile, has been unequivocally established in Southwestern Indians. The reversal of this abnormality could lead to both the prevention and the dissolution of gallstones.

Proposed Course:

It has now been demonstrated that the administration of chenodeoxycholic acid to persons with lithogenic bile and gallstones can result in conversion to non-lithogenic bile and dissolution of the gallstones.

Confirmation of these findings in a controlled manner and carefully controlled observation of the efficacy and/or possible side effects of this mode of treatment are urgently needed.

Honors and Awards: None.

Publications: None.

Serial No. NIAMD-EFS-5(c)
1. Epidemiology and Field Studies Branch
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Prospective Study of the Complications and Outcome of Diabetic and Prediabetic Pregnancies in the Gila River Indian Community

Previous Serial Number: NIAMD-EFS-6(c) (1971)

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Dr. S. A. Kamenetzky
Dr. Arthur Dorf
Dr. Max Miller

Cooperating Units: Indian Health Service (PHS)
Case Western Reserve University

Man Years:

Total:	0.7
Professional:	0.2
Others:	0.5

Project Description:

Objectives:

To determine whether pregnancies in prediabetic women are associated with excessive complications during pregnancy or with excessive mortality or morbidity in the offspring.

Methods:

The course and outcome of pregnancies in all female residents of the Gila River Indian Community (Sacaton Service Unit area) will be followed and recorded in a standardized manner.

Infants will be examined to determine the presence of congenital anomalies and other characteristics, and data related to mortality and morbidity will be compiled. The mothers will be given modified glucose tolerance tests 24-48 hours post partum and retested at approximately two yearly intervals to document the development or lack of development of diabetes. At age five years the children will be re-examined to detect any further abnormalities which may have been unapparent at birth.

Major Findings:

None. Further analysis has been deferred pending collection of additional data.

Significance of Research:

Retrospective studies of the characteristics of prediabetic pregnancies have yielded conflicting results. The study of pregnancies among the Pima Indians, who have a high prevalence of diabetes mellitus, provides a unique opportunity to evaluate a larger number of prediabetics prospectively.

Proposed Course:

The pregnancy study will continue in conjunction with the study on the natural history of diabetes mellitus in the Gila River Indian Community. The number of pregnancies recognized to be prediabetic will increase as the mothers studied develop diabetes mellitus.

Honors and Awards: None.

Publications: None.

Serial No. NIAMD-EFS-6(c)
1. Epidemiology and Field Studies Branch
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Gila River Indian Community Autopsy Study

Previous Serial Number: NIAMD-EFS-7(c) (1971)

Principal Investigator: Dr. Peter H. Bennett

Other Investigators:
Dr. Joseph Likos
Dr. S. A. Kamenetzky
Dr. J. A. Ingelfinger
Dr. Arthur Dorf
Dr. Philip M. LeCompte
Dr. Max Miller

Cooperating Units:
Indian Health Service (PHS)
Veterans Administration
Faulkner Hospital, Boston, Massachusetts
Case Western Reserve University

Man Years:

Total:	1.0
Professional:	0.3
Others:	0.7

Project Description:

Objectives:

1. To determine the causes of death, and associated disease processes in members of the Gila River Indian Community being followed in prospective studies on arthritis, diabetes mellitus, cholelithiasis, etc.

2. To ascertain and evaluate the histopathological changes in tissues and cells as they relate to diabetes mellitus. To assess their importance and relationship to clinical findings in members of the Gila River Indian Community upon whom autopsies are performed.

3. Evaluate factors and conditions associated with increased or decreased mortality rate in the Gila River Indians.

Methods:

1. Permission to perform a postmortem examination will be sought from next of kin of all members of the Gila River Indian Community who die in the Sacaton Hospital or Phoenix Indian Medical Center.

2. The autopsy will be performed in a meticulous manner with special emphasis on examination of tissues and organs believed to be affected in diabetes mellitus.

The tissues will be examined using routine and special staining techniques and by light and electron microscopy when necessary.

3. The information will be correlated with the course/progression of clinical abnormalities studied in the same individuals during life.

Major Findings:

Autopsy Evidence of Myocardial Infarction: Autopsy results of 100 Pima Indians, aged 40 and over, examined between 1/1/65 and 1/1/72 were reviewed. Eleven myocardial infarcts were found as judged by gross myocardial scarring and confirmatory histological examination.

AUTOPSY EVIDENCE OF MYOCARDIAL INFARCTS

	Males		Females	
	Known Diabetics	Non-Diabetics	Known Diabetics	Non-Diabetics
No. Examined	20	35	15	20
With Infarcts	6(30%)	4(11%)	0(0%)	1(5%)

The number of autopsies is still small and interpretation of trends is made more difficult by the fact that the selection factors in the autopsy population are ill defined.

Deaths Due to Coronary Heart Disease: Seventy-six diabetic Pima males, age 40-59, on initial examination have been followed for a mean of 5.4 years. Clinical record review indicated that there were seven deaths in this group, one of which was caused by myocardial infarction.

The age standardized death rate was 215 per 10,000 men per year, with 30.5 per 10,000 men per year due to myocardial infarction. The age standardized death rate for U.S. Railway workers, age 40-59, has been reported as 94 per 10,000 men per year, with coronary heart disease accounting for 47 deaths per 10,000 men per year.

Diabetic Nephropathy: Renal tissues obtained from unselected postmortem examinations from Indians of the Gila River Community were reviewed and graded for the presence or absence of mesangial changes, nodular glomerulosclerosis, exudative changes, arteriolar narrowing, and pyelonephritis. Forty percent of the subjects were diabetic. Of these, two-thirds had evidence of moderate or severe mesangial thickening. Of those with mesangial disease, 75 percent had nodular glomerulosclerosis as well. None of the patients had nodular involvement without evidence of mesangial disease as well. The mean duration of known diabetes in those with both diffuse and nodular glomerulosclerosis was 12 years, whereas the duration of diabetes in persons without mesangial changes averaged 5.5 years.

Significance of Research:

This study will result in a more precise clinical-pathologic correlation than has been previously possible in diabetes mellitus. It will enable the prognostic significance of a variety of clinical findings to be assessed. The obvious advantages of an epidemiologic approach will be realized and a more complete understanding of the significance of glucose intolerance and its sequelae will result.

The prevalence of coronary heart disease at death will be related to the prevalence and incidence of coronary heart disease in life. This will determine whether the low prevalence in life reflects a poor prognosis or whether coronary heart disease is truly an infrequent disease in the Pima Indian.

The observation of a high prevalence of histologic evidence of the specific renal changes of diabetes will enable this disorder to be characterized more fully both clinically and histologically.

Proposed Course:

The systematic collection of tissues will continue. As sufficient specimens become available for special study, detailed examinations of various tissues will be made. Further studies of the kidney, using electron microscopy are planned.

Honors and Awards: None.

Publications:

Bennett, P.H., Miller, M., Burch, T.A., Ballintine, E.J., and LeCompte, P.M.: Hyperglycemia and the specific retinal and renal complications of diabetes in the Pima Indians. In Reports on Oral Diabetes Therapy Especially with HB 419, Seventh Congress International Diabetes Federation (1970). Amsterdam: Excerpta Medica Foundation, 1971, pp. 20-25.

Serial No. NIAMD-EFS-7(c)
1. Epidemiology & Field Studies Branch
2. Metabolic Diseases Epidemiology Unit
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Experimental and Field Studies of Iodine Metabolism

Previous Serial Number: NIAMD-EFS-8(c)

Principal Investigator: Robert L. Vought, M.D., M.P.H.

Other Investigators: Freddie A. Brown, B.S.

Cooperating Units: Laboratory of Nutrition and Endocrinology, Clinical Endocrinology Branch, NIAMD; Theoretical Statistics and Mathematics Unit, Biometry Branch, NIMH; and Richmond County Virginia Health Department.

Man Years:

Total:	3
Professional:	2
Other:	1

Project Description:

Objectives: The long-term objectives of the program are: (a) to examine closely the principal environmental factors associated with prevalence of goiter and (b) to study the nature of the metabolic defects responsible for the disease.

Methods Employed: Accomplishment of the objectives has involved bench laboratory research, clinical research and collaborative field studies.

The intensive microbiologic studies of a well associated with high goiter prevalence have now been completed. Approximately 60 micro-organisms from the well and 10 from other sources were screened for goitrogenic activity. Routine chemical, microbiologic and pharmacologic studies continued as before.

Major Findings: Goitrogenic activity was detected in cell-free, lyophilized filtrates of broth cultures in the following organisms. Penicillium, four species, two from the well and two from rat diet, were consistently positive. The other most promising isolate from the well was Desulfovibrio desulfuricans. Other organisms showing some goitrogenic activity were two strains of Escherichia coli, a strain of Neisseria, Bacillus sp., Alcaligenes sp. and a Proteus sp. showed questionable activity. Present attention is focused on the determination of the culture conditions which give rise to optimal goitrogen production.

Further work on iodide excess in the United States was done and the results presented at a national conference on trace substances. It has now been verified that average daily iodine intake is approximately 400 μg, a figure which we reported in 1964. This has now been verified by other investigators using different techniques for estimation of intake. It also appears that these figures are increasing due to iodine entering the diet from adventitious sources, e.g. erythrosine, which is widely used in coloring food and pharmaceuticals. The latter finding, done in cooperation with Clinical Endocrinology Branch, has been reported. Assistance to the Nutrition Program, Center for Disease Control in Atlanta was continued and their findings may shed more light on iodide consumption and goiter prevalence.

Publications:

Vought, R.L., Brown, F.A., Sibinovic, K.H., and McDaniel, E.G.: Effect of changing intestinal bacterial flora on thyroid function in the rat. Horm. Metab. Res. 4: 43-47, 1972.

Vought, R.L., Brown, F.A., and Wolff, J.: Erythrosine: An adventitious source of iodide. J. Clin. Endocr. 34: 747-752, 1972.

Vought, R.L.: Upward trend in iodide consumption in the United States. Proc. Fifth Annual Conference on Trace Substances in Environmental Health, June 29-July 1, 1971, University of Missouri, Columbia. (In press)

OFFICE OF PUBLIC INFORMATION ACTIVITIES

Summary

Fiscal Year 1972

This past fiscal year saw the establishment of an Office of Public Information in the Office of the Director. The functional description of this Office is as follows:

> "Advises the Director on public information, public education, and public relations aspects of the Institute's program; collects, develops and disseminates information on Arthritis and Metabolic Diseases using various media; collaborates with voluntary and private organizations in planning public information and education programs."

As a component Federal agency, NIAMD is accountable to the American people. It must report to the people on its stewardship of the programs entrusted to it. Thus the primary objective of information activities at NIAMD is to increase this kind of public knowledge, understanding, and awareness.

There are not one but several publics which must be reached with NIAMD information. They can be grouped as follows:

The general public, itself made up of several subdivisions: informed laymen, students and parents, potential health careerists, minority and disadvantaged groups, civic and voluntary health organizations.

The professional public, which includes: medical and other health practitioners; scientists, professional associations, and scientific organizations; and academic institutions. A special and sizable group within the professional public includes grantees, contractors, and collaborating institutions.

The Governmental public: the internal audience within NIH and HEW: other Federal agencies; state and local governments. The NIH employs an extensive advisory structure in administering its programs, and these advisory groups constitute a special audience which can most logically be included in this category.

The Congressional public: members of Congress, their staffs, and staffs of Congressional committees.

The media we use fall into three broad categories:

Mass Media--suitable for general messages and campaigns aimed at the widest possible audience. These media consist of: newspapers, magazines, radio, television, publications, and films.

Specialized Media--used for more specific, targeted approach. These media include: professional journals and newspapers, conferences, speeches, and exhibits.

Individual Communications--mail, telephone, personal contacts, direct mail efforts. Individual communications are used primarily to respond to requests for information or to reach highly specialized groups.

The following is a review of the performance of the Office of Public Information during the past fiscal year.

Press Activities:

It is the policy of the National Institutes of Health to prepare and issue news releases on newsworthy subjects about its research activities and programs. In this regard, the NIAMD information office staff this past fiscal year prepared information on a wide variety of topics.

Characteristic press releases included the following: (1) the fact that experience gathered from managing epidemics of Asiatic cholera, a devastating form of diarrhea, is being applied to treatment and prevention of acute diarrheas of certain American Indian infants; (2) the establishment by the NIAMD of two new training awards, the Clinical Investigator Award, and the Academic Career Development Award, both in Digestive Diseases and Nutrition; (3) announcement of the availability of a new publication entitled "Kidney Disease and Artificial Kidneys," which was written by an information office staff member, and (4) the announcement that Dr. Robert L. Sinsheimer of the California Institute of Technology would deliver the 1972 National Institutes of Health Lecture.

Television and Radio:

The information arm of the Office of the Director has participated with the central NIH information office in the production of projects designed to bring the Institute to the attention of the public. These projects include:

NIH Reports--this weekly television segment on WRC-TV and certain of its affiliates has featured a number of NIAMD intramural and extramural personnel in five minute interviews. The show is telecast on Sunday afternoons.

Discussion: NIH-Every Friday during the intermission of the Library of Congress chamber music concert an NIH staff member takes part in a 15-minute interview on WGMS radio. A number of NIAMD staffers have appeared.

In addition to the above, experts from NIAMD have appeared on Channel 5's Panorama, among other television and radio shows.

Special Events:

The IV International Congress of Endocrinology, sponsored by the International Society of Endocrinology, took place in Washington, D.C. June 18-23. Drs. J. E. Rall and G. D. Aurbach were chairman and secretary-treasurer, respectively, of the organizing committee.

Information activities included the preparation of press releases and special background material for science writers. In addition, information office staff members manned the Congress' press room during the six-day meeting. This successful information effort resulted, in part, from close cooperation and liaison work with publicity representatives of the International Society of Endocrinology.

The effectiveness of NIAMD information involvement in this international meeting was measured by the gratifying response of scientific and paramedical journals and the lay press, whose coverage of the meeting was based largely on materials prepared by the information staff.

Publications and Reports:

Three separate NIAMD publications either were undergoing revision or preparation during FY 1972. The information office's most recent effort involves the preparation of a pamphlet about gallbladder disease, a disease area in which public interest has been stimulated in recent years. Undergoing revision are a fact sheet on hypoglycemia, which has been well received in the past, and the Institute's most popular publication "Facts About Nutrition."

During the past fiscal year the Institute gave impetus to its Equal Employment Opportunity program. In this regard, much time was spent by Information Office staff members towards the development of the Affirmative Action Plan.

The so-called "weekly report" forms the basis for the bulk of the regular and special program reports required of the Institute by the Congress, DHEW, and NIH. Such reports are based upon intramural or extramural research accomplishments. Fiscal Year 1972 was an unusually active year for weekly reports because of many significant findings in both basic research and clinical studies.

Typical weekly reports in FY 1972 included such developments as the dissolution of cholesterol gallstones by medical means; synthesis of luteinizing hormone-releasing hormone and its use in inducing ovulation in animals; the demonstration that phenformin is of dubious value in the long-range management of diabetes, except insofar as it lowers elevated blood sugar levels; assessment of the clinical, microbiologic,

and morphologic features of experimentally induced Mycoplasma hyorhinis arthritis (which resembles human rheumatoid arthritis) in swine, and the demonstration that vitamin D is converted in kidney tissue to the ultimate biologically active form of the vitamin.

Public Inquiries:

A major and most important function of the information office is answering public inquiries which come from a wide spectrum of populations, ranging from people with illnesses, and school children with science fair projects, to congressmen, scientific researchers and practicing physicians. Considerable staff time is involved in handling such requests, often at savings to the time of NIAMD scientific and administrative personnel. Inquiries are often quite specific and may require information that is not readily available.

During Fiscal Year 1972, the information office responded to 2,510 public inquiries, 180 Congressional inquiries, and 91 inquiries from representatives of the various news media. These requests for information are usually made either by letter or by telephone, and information office staffers usually respond in kind. Also, during FY 1972, the information office distributed an unprecedented number of publications, approximately 280,000. This figure represents an increase of almost 100 percent over that of Fiscal Year 1971.

When the Diabetes and Arthritis Control Program, Division of Chronic Diseases, was phased out, the information office was distributing about 10,000 publications each month. With the added responsibility of replying to the defunct agency's correspondents, this figure now approaches 20,000 per month.

Exhibits:

The information office also conducts the NIAMD program of scientific exhibits. This is a prime source of professional education. NIAMD displayed five different exhibits during FY 1972 at 9 medical and scientific meetings throughout the country, which were viewed by approximately 70,000 physicians, scientists and paramedical people. Institute exhibits on human growth hormone, artificial kidney, cystic fibrosis, arthritis and other research activities were among those shown. Institute publications and reprints are distributed in conjunction with these exhibits. Information office staff members, including the exhibit specialist, all write press releases, reports, etc., as needed. In this manner the NIAMD information office can maintain a substantial and varied output with 8 members, including clerical staff, compared to 15-20 in some of the other Institutes.

OFFICE OF THE DIRECTOR

Summary

Fiscal Year 1972

Dr. G. Donald Whedon, Director
Mr. W. G. Baylis, Executive Officer

The Office of the Director has overall responsibility for directing and coordinating basic laboratory research; clinical investigations; extramural activities such as research grants, research fellowships, and training grants; contract research and developmental programs; and epidemiologic and clinical field studies. In addition, this Office cooperates with other scientific organizations in coordinating medical research in the Institute's fields of interest; cooperates and maintains liaison with other Institutes and constituents of other Federal agencies; and participates in determining policy governing the National Institutes of Health. It is also responsible for the collection and dissemination of informational material to interested professional and lay individuals and groups.

The continuing review of the Institute's organizational structure to better identify the areas of responsibility has resulted in the establishment of new Offices and Sections. Change of the names of some Sections were also made to reflect more properly the scope of their functions and responsibilities. The Pilot Plant Operation, Section on Developmental Biochemistry and the Germ Free Research, Section on Vitamin Metabolism were moved into the Office of the Chief, Laboratory of Nutrition and Endocrinology. A new section on Theoretical Molecular Biology was established in the Laboratory of Molecular Biology and the name of the Section on Chemical Genetics, Laboratory of Molecular Biology was changed to Section on Molecular Genetics. The name of the Section on Histochemistry, Laboratory of Experimental Pathology, was changed to Section on Molecular Pathology. The Section on Biochemical Regulation, Laboratory of Biochemical Pharmacology was abolished. In the Extramural Program area the Gastroenterology Program was renamed the Digestive Diseases Program, and the Urology and Renal Disease Program was renamed the Kidney Disease and Urology Program.

Several changes have been made in the staffing of some of the key positions within the Institute during this fiscal year. Dr. Lionel M. Bernstein was appointed Acting Assistant Director for Digestive Diseases and Nutrition; Dr. David F. Johnson was appointed Chief, Section on Microanalytical Services and Instrumentation, Laboratory of Chemistry; Dr. H. Todd Miles was appointed as Acting Chief, Laboratory of Molecular Biology; Dr. Juni-ichi Tomizawa was appointed Chief, Section on Molecular Genetics, and Dr. Terrell L. Hill was appointed Chief, Theoretical Molecular Biology Section.

The Office of Public Information was established in December 1971; Mr. Victor Wartofsky was appointed Chief. The activities of this Office are included in

a separate report which precedes this summary. As in previous years, this Office has continued its efforts aimed at informing the general lay public of the Institute's mission and activities. Response was made to more than 2,510 inquiries from the public. Five different scientific exhibits were displayed at nine medical and scientific meetings throughout the country which were viewed by approximately 70,000 physicians, scientists and paramedical people, and over 280,000 publications were distributed.

Also, in December 1971, The Office of Administrative Management was established and Mr. W. G. Baylis, as Executive Officer, was appointed its Director. The functional duties of this Office are as follows:

> "Advises the Director on administrative matters; plans and directs management functions of the Institute including budget, financial management, contract award and management, personnel, procurement, office services, management analysis, etc.; interprets, analyzes, and implements new legislation, administrative orders, and new concepts affecting the overall mission of the Institute."

Mr. William A. Carr was appointed as the Institute's Contracting Officer. The decentralization of contracting authority to the Institute results in absorption of all of those functions previously performed by the central Research Contracts Branch. Even with the problems associated with this type of transition, the operating relationships and coordination within the Institute and with other elements such as the Office of Contracts and Grants, Financial Management, DHEW Patent Advisors, and the contracting community, have continued to be good. The volume of activity continued through Fiscal Year 1972 with some increase in the number of active contracts (from 80 to approximately 90). These contracts were for Artificial Kidney development, hormone distribution, scientific communications, and augmentation of intramural research. In addition, the last quarter of the fiscal year saw the emergence of new programs involving the contract mechanism. These were: (1) Gastroenterology and Digestive Diseases; and (2) Kidney Diseases. Under the first program, one major contract was awarded, involving eight participating subcontractor centers for the clinical evaluation of the efficacy of three drugs in the treatment of Crohn's disease under controlled protocol conditions. Steps were also taken which ultimately will lead to the award of a similar multi-center evaluation of methods for treating gallstones.

A special program evaluation project was also initiated, and steps were taken which will result in the award of several contracts to measure the impact of NIH research and training support on the quality of patient care service provided by institutions receiving such support. This is planned in a two-phase project, the first providing for design of the study protocol, and the second providing for conduct of the actual study. It is anticipated that the total project will encompass a period of several years.

New applications for use of the Wylbur text editing computer program and remote computer job entry were initiated during the past year. One new major application is the use of Wylbur to link from NIH's ARMS system to secure personnel data required by the Institute. This data included monthly staff

lists, ceiling contral and grade escalation summaries and special personnel reports from 19 data fields.

Management continues to place emphasis on enhancing training opportunities offered both in-service and through non-governmental and other than HEW governmental facilities. Part IV of the Basic Ideas in Biology has been completed by the Institute's Personnel Office. Another new program, Basic Ideas in Biochemistry will be initiated later in the year. These training programs are part of the Institute's overall effort to increase employee productivity and to fully utilize employees with potential for advancement in career fields related to their interests and abilities. During the year short-term training opportunities were arranged for 176 Secretarial-Clerical employees, 4 supervisory managerial personnel and 74 members of the professional staff.

Equal Employment Opportunity

The Institute held an Equal Employment Opportunity (EEO) meeting in the Jack Masur Auditorium for all NIAMD employees at which time the chairmen of the subcommittees of the NIAMD Committee on Equal Employment Opportunity reported the progress and plans for each group. This group also met with the Laboratory/Branch Chiefs at one of their regular monthly meetings and with the NIAMD Assembly of Scientists and at staff meeting of the extramural program employees. The monthly meetings of black and women employees are continuous. An EEO Newsletter has been started for distribution to all employees of the Institute keeping them informed on items of interest and progress made in the EEO Program within NIAMD. This is a continuing publication and is published as items of interest arise.

An EEO Retreat was held in Baltimore, attended by approximately 70 employees. Discussions at this conference produced over 50 recommendations for action that were presented to the NIAMD EEO Committee for consideration. These recommendations were discussed in detail by the Committee and an NIAMD Affirmative Action Plan has been published and distributed to all Institute employees. This publication lists all recommendations made and accepted and identifies the responsibility for implementation of each of the recommendations. Many of these recommendations have either been implemented or implementation has begun.

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